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Fluorescent-Antibody Techniques in Diagnostic Bacteriology[1]

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INTRODUCTION

The technique of immunofluorescence as developed by Coons et al. (10, 11) was detailed and extended by Coons and Kaplan (12). Since that time, it has proved to be a powerful tool for immunological research, experimental pathology, cytological investigations, and diagnostic microbiology.

It is the diagnostic area which is the subject matter of this review, or, more precisely, the role of the fluorescent-antibody (FA) technique in bacteriology. Approximately 8 years have passed since the appearance of the first publications dealing specifically with this topic. These were a series of two papers by Moody et al. (45) and Thomason et al. (77) on the rapid detection of *Malleomyces mallei* and *M. pseudomallei* in smears prepared from cultures, animal tissues, and environmental specimens stained with a specific conjugate of *M. pseudomallei*.

No attempt will be made in this review to discuss all of the proposed applications of immunofluorescence to diagnostic situations in bacteriology. Several of these are rapid, sensitive, and reliable, but are of minor interest due to infrequency of occurrence of the disease for which they were designed. Tests for some systems are limited by lack of serological specificity, inadequate evaluation, or other factors. A few of the more important miscellaneous applications of FA tests will be tabulated and discussed briefly.

No useful purpose can be served by enumerating all of the bacterial species that have been observed to fluoresce when exposed to homologous, labeled antibody. In fact, we are not aware of any, including the acid-fast bacteria, that will not give immunofluorescence, the only requirement being an exposed antigen or haptene at the surface of the bacterial cell. It is as true today as it was 8 years ago that each new application of the FA technique poses special problems. Each new test requires its own field evaluation. This

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situation exists primarily because of the inherent nature of immunofluorescence techniques—extraordinary sensitivity, theoretically and often in practice, permitting the detection or identification of a single bacterial cell admixed with large numbers of contaminants. Usually no difficulty is experienced in locating specifically stained bacteria in mixtures containing ratios of contaminants to specific cells as high as $10^7:1$ (77).

At the time immunofluorescence techniques were introduced, information available on serological relationships existing among organisms constituting the normal flora of the intestinal tract or of the nasopharynx was inadequate to permit determination of the extent to which this would be a problem in FA studies. Shared antigens were of minor importance when identification of a particular species of bacteria was based upon isolation and characterization of the organism in pure culture. Immunofluorescence of bacteria has only two dimensions—morphology and serological specificity. The specificity of an FA test applied to mixed flora, therefore, must be determined experimentally for each bacterial species sought. Lack of specificity can be tolerated only if the morphology of the cross-reacting antigen clearly distinguishes it from the specific antigen. For example, there is no way in which the reliability of an FA test for *Salmonella typhosa* can be measured short of proving that (i) it will detect this species in the feces of bacteriologically proved carriers or cases, and that (ii) it will not give positive results on fecal specimens from persons bacteriologically free from this organism. In no other way can one be sure that the gut does not contain unrecognized or unculturable bacteria or artifacts morphologically and serologically resembling *S. typhosa*.

Our purpose is to review critically those applications of immunofluorescence in diagnostic bacteriology that are judged of greatest importance at the present time.

MOST IMPORTANT DIAGNOSTIC APPLICATIONS OF IMMUNOFLUORESCENCE

Identification of Group A Streptococci

Without doubt, the most widely used diagnostic FA test is that for the identification of group A streptococci. The basic data were developed in a paper by Moody et al. (46), who showed that conjugated globulin for group A streptococci, when adequately sorbed and diluted, could be used to differentiate these organisms from those of all other groups (A to G) that were tested. Cross-staining of streptococci of group C could be eliminated by sorption of the group A conjugate with streptococcal cells of group C. Smears of cultures and clinical specimens were prepared on slides, dried, fixed, and stained. These preliminary results were judged to be quite encouraging in terms of rapidity, specificity, and economy. Isolation and grouping of streptococci by the precipitin technique usually requires 3 to 5 days as compared with a few hours to less than 1 day by immunofluorescence.

Subsequently, an extensive field evaluation of the technique was undertaken in which FA and cultural-precipitin procedures were compared for the identification of group A streptococci obtained from paired nasopharyngeal swabs taken simultaneously from patients in the Chicago, Ill., area (49). One swab was examined by conventional cultural methods; the other, by FA techniques. Two smears were prepared from each specimen, one being exposed to the specific FA reagent and the other to the control reagent. Cotton was compared with dacron as a fiber for collection of specimens. The results of direct examination of the nasopharyngeal specimens by both cultural and FA procedures were compared with results obtained by applying the same methods after preliminary (2 hr) enrichment of the swab specimens in a suitable broth medium.

The FA reagents employed consisted of group A conjugate sorbed with group C cells and, as a control on each specimen examined, normal rabbit conjugate sorbed with group A cells. The latter served as a control on the specificity of staining, because any normal antibody for group A streptococci had been removed. Thus, an FA reaction with the group A conjugate and a negative one with the sorbed normal conjugate constituted a positive FA test for group A streptococci. Conversely, stained organisms in both smears indicated a heterologous reaction usually due to staphylococci.

It is quite clear from the work reported in the above paper that direct smears were quite unsatisfactory for detection of group A streptococci by FA staining when compared to either direct culture or to cultural or FA techniques applied to broth enrichments (Table 1).

The significance of the differences in the number of detections from cotton as compared with dacron swabs by either of the two methods used is not clear, since factors such as swab size and absorbancy may be as important as possible toxicity of cotton fibers.

When the results of testing 482 paired swabs by direct conventional culture methods followed by precipitin grouping were compared with results obtained by FA staining of 2-hr broth enrichment cultures, only 75 (38%) proved positive by culture, and 172 (88%) were FA positive. When specimens initially positive by

FA but culturally negative were re-examined by repeated plating, group A streptococci were recovered from almost one-half. The group of specimens yielding positive cultures but negative FA staining reactions were re-examined by the staining of one additional smear. In every case group A streptococci were detected.

The superiority of the enrichment procedure over conventional direct plating for detection of group A streptococci by either the cultural-precipitin or FA methods was a significant finding. It was postulated that the reasons could be (i) multiplication of the streptococci or (ii) removal of mechanical or chemical inhibitors of growth or staining. The question of the significance of detecting small numbers of group A streptococci in nasopharyngeal exudates after some of the positive reactions obtained by direct FA examination represent staining of staphylococci, since adequate controls did not appear to have been included. Experience has shown that morphology alone is inadequate for differentiation of staphylococci from streptococci in many cases.

Wolfe and Cameron (92) reported the first large-scale evaluation of the grouping of streptococci by immunofluorescence. They employed conjugates prepared by Moody to stain smears prepared directly from field cultures consisting of throat swabs inoculated onto Trypticase Soy Agar (BBL) slants. Specimens taken by physicians were mailed to the laboratory. The FA procedure made it possible for the submitting physician to receive a preliminary report within

TABLE 1. *Effect of broth enrichment on identification of group A streptococci by FA and culture-precipitin grouping tests**

Kind of swab	Methods compared	No. examined	Per cent positive	
			Direct	Broth
Cotton	*FA*			
	Direct vs. 2-hr broth	63	3	46
	Conventional			
	Direct pour streak vs. 2-hr broth pour streak	63	18	33
Dacron	*FA*			
	Direct vs. 2-hr broth	163	11	37
	Conventional			
	Direct pour streak vs. 2-hr broth pour streak	164	24	41

* Taken from Moody et al. (49); copyright 1963 by the American Public Health Association, Inc.; reprinted with permission.

preliminary broth enrichment is the province of the clinician.

When all patients who were positive for group A streptococci by either method used in the study were considered, it was found that 81% were detected by FA, and 74% were positive by culture. The agreement between the two tests on both positive and negative specimens was 80%. There was no indication of lack of specificity of the group A antibody in the conjugate used in this study.

The findings obtained by Warfield et al. (86) with the methods recommended by Moody et al. (46) are at variance with the latter in respect to the efficiency of 2-hr broth enrichment for enhancing the detection of group A streptococci. The former workers studied a series of 500 specimens, 96 of which were FA positive by direct staining. Only 67 of these were culturally positive after broth enrichment. Although they used a group A conjugate sorbed to remove group C *Streptococcus* antibody, it is conceivable that 3 days instead of the usual 7 to 10 days. Comparative cultural and FA examination of 722 specimens showed an agreement between the two methods of 89.2%. The latter method was considerably more sensitive, presumably due to the examination of a larger fraction of the specimen by FA staining than was possible by culture on blood-agar. Routine culture methods revealed 29.5% positive specimens, and 36.4% were positive by immunofluorescence. Only 1.3% of all specimens positive by culture were negative by FA staining, but 8.8% positive by FA were negative by culture.

Each specimen was stained by both group A conjugate sorbed with group C cells and by a labeled, normal, rabbit conjugate sorbed with group A cells. In only five specimens were fluorescent cells observed in both the test and the control smears. Coagulase-positive staphylococci that were stained by the conjugates were isolated from four of these specimens. Wolfe et al. (93) extended these studies to include a total of

2,719 specimens examined for hemolytic streptococci by both FA and cultural methods. Agreement between the two methods occurred in 90.2% of the specimens; 34% of the total were positive by one method or both. Only FA staining occurred in 21.3% of the total number of positive specimens, and group A streptococci were obtained by culture in the absence of positive FA reactions in 2.4%. The pertinent findings in this extensive study are summarized in Table 2.

Associated with the above study was an evaluation of dry swabs versus swabs inoculated onto Trypticase Soy Agar as a mechanism of transport of throat specimens for detection of group A streptococci by FA procedures. Duplicate throat swabs, one of which was placed in a sterile tube, the other inoculated onto a Trypticase Soy Agar slant, were mailed to the laboratory. The results indicated that dry throat swabs submitted in this way were not as satisfactory as the cultured specimens for detection of group A streptococci by the FA technique. False positive staining due to staphylococci did not interfere appreciably with the application of the FA staining procedure to streptococci.

TABLE 2. *Results obtained from throat cultures using FA conjugates and blood-agar culture methods**

Determination	No. of specimens	Per cent of total positives	Per cent of total specimens
Total specimens tested	2,719		100.0
Negative both for β-hemolytic streptococci and with group A conjugate	1,794		66.0
Total positive either method	925	100.0	34.0
Total agreement of both methods	2,453		90.2
Positive for β-hemolytic streptococci	728	78.7	26.8
Positive with group A conjugate and negative by culture	197	21.3	7.2
Positive with group A conjugate	856	92.5	31.5
Negative with group A conjugate and positive by culture†	69	7.5	2.5
Positive by culture but not belonging to group A when precipitin-typed	40	4.3	1.5
Positive by culture, group not determined	7	0.8	0.3
Group A not detected with conjugate	22	2.4	0.8

* This table is from an article by Wolfe et al. (93) and was reprinted with permission. The table includes 722 specimens previously reported by authors. All examinations recorded were made in Central Laboratory, Tennessee Department of Public Health, from 2 February 1959 through 18 August 1960.

† Two isolations, one a group B and the other a group C *Streptococcus*, were made from a single specimen, thereby making 70 isolations from 69 specimens.

Since that time, several investigators have called attention to the frequent occurrence in fluorescein-conjugated animal sera of antibodies directed against staphylococci (8, 9, 59, 60, 63, 64). Control of this factor has been an important aspect of the specificity of the FA procedure for grouping of streptococci.

Redys et al. (63) evaluated the fluorescence technique for grouping of streptococci in a busy state health laboratory where, to be useful, such a procedure must give results equal or superior in reliability to those of classical procedures and must result in monetary savings. They devised a procedure for inhibition of the common antigen fluorescence given by groups C and G streptococci when exposed to group A conjugate. The essence of this technique is the application of group C antiserum to smears prior to use of the group A conjugate. Usually it is desirable to sorb the latter with group C cells prior to use. This approach to the problem of blocking undesirable cross-reactions was combined with the enrichment of the throat specimens in Streptosel broth (BBL) to inhibit the growth of coagulase-positive staphylococci that react with normal globulins. When the sensitivity of detection of group A streptococci in smears from Streptosel broth was compared with that of plating on blood-agar, it was found that only 78% of the specimens positive on blood-agar also proved positive from the broth. However, the latter, even though less sensitive for detection of streptococci, was quite effective in eliminating coagulase-positive staphylococci which were found on primary blood plates from 11.2% of 223 consecutive throat-swab specimens.

Redys et al. (63) examined 4,480 throat swabs, 1,256 of which yielded β-hemolytic streptococci on blood-agar. The reactions of isolates from 1,239 of these specimens were compared by precipitin grouping and by the "inhibition-of-common-antigen fluorescence" procedure which they described. The two methods proved

equally reliable for detection of group A streptococci.

Subsequently, Redys et al. (64) modified the test used earlier so that a one-step procedure could be employed to inhibit common antigen fluorescence due to group C and G streptococci. Fortunately, the group C serum also reduced or eliminated the cross-staining of staphylococci due to its content of normal antibody for the latter organisms. To avoid the examination of specimens negative for group A streptococci, these workers inoculated both blood plates and an appropriate broth medium with the primary specimen. After overnight incubation, smears were made from the broth culture of specimens from which corresponding blood plates revealed β-hemolytic colonies. This procedure reduced the work load of the laboratory by approximately one-half, because FA grouping was performed only on culturally positive specimens. This one-step inhibition test effectively eliminated cross-reactions with staphylococci as well as with group C and G streptococci; therefore, it was not necessary to do parallel FA testing with normal rabbit conjugate. It was shown in the examination of 1,534 specimens that the screening of throat swabs for β-hemolytic colonies on blood-agar plates and grouping by FA the corresponding broth cultures gave false negative results in only 0.6% of the specimens examined. This simplified test is now used routinely in a state laboratory processing from 100 to 700 specimens daily, with an annual volume of 30,000 to 50,000 specimens (E. K. Borman, *personal communication*). Another state performed similar tests on almost 17,000 specimens during 1962–1963 (G. M. Cameron, *personal communication*). The same state reported results on 4,000 specimens during the single month of February, 1964. A third state grouped more than 16,000 cultures of human streptococci by the FA technique during 1963 (C. D. McGuire, *personal communication*). It is not a coincidence that this application of immunofluorescence also has received an intense promotional campaign sparked by the Heart Disease Control Program of the U.S. Public Health Service and by state and local groups interested in the prevention of rheumatic fever and other sequellae of *Streptococcus* infection. More people have received formal intensive laboratory training in the identification of group A streptococci by immunofluorescence than for any other application of this test. Several years of experience have proved that 2 weeks of intensive course work is sufficient for training the average public health laboratory bacteriologist to group streptococci from throat swabs with a high degree of reliability by use of immunofluorescence. Data summarized in Table 3 were compiled from reports received from 58 state and metropolitan public health laboratories and consist of the results of evaluation studies performed in their respective facilities after the training period. A total of 32,460 paired throat swabs were examined, one by culture-precipitin, and one by FA methods. Of these, 7,401 were positive by both methods and 23,290 were negative by both, giving a 94% agreement. Of 8,478 which were positive by both methods, 92% were detected by FA methods and 88% by the culture-precipitin tests.

Additional validations of the FA technique for identification of group A streptococci were reported by Peeples et al. (59). In processing two groups of specimens, one submitted on the original swab and the other dried on filter-paper strips inoculated from the swab, agreement between the FA and cultural methods performed on 2- to 4-hr broth cultures inoculated from the above materials was better than 95%. Their field study showed that the FA technique was rapid, accurate, and practical for use in any public health laboratory.

TABLE 3. *Cummary of comparative evaluation of FA and culture precipitin tests for detection of group A streptococci by state laboratories*

Culture precipitin	FA		Totals
	+	−	
+	7,401	692	8,093
−	1,077	23,290	24,367
Totals	8,478	23,982	32,460

Scott and Holloway (65) examined throat swabs from 100 patients suspected of having group A streptococcal pharyngitis by using both cultural and FA staining methods. They commented that the FA procedure proved to be at least as sensitive and far more rapid than conventional cultural methods. In another comparative study involving the processing of 800 specimens from diverse sources of group A streptococci, the FA procedure proved quite satisfactory (85). However, Wagner and Heinrich (85) called attention to the fact that certain strains of streptococci belonging to groups B, C, and G gave fluorescent reactions with labeled antibody prepared with a type 28 strain of group A or with sera containing the T2 antibody found in some group A strains. The significance of this observation is uncertain due to their use of labeled whole serum at low dilution Apparently,

the above-mentioned cross-reactions occurred even though the conjugated antiserum was sorbed with group C cells and with hog-kidney powder.

In another study of a total of 1,115 throat cultures from children in Miami, Fla., the investigators employed five modifications of the FA technique and compared these, for efficiency in the identification of group A streptococci, with three streak-plate techniques, a pour-plate procedure, and the conventional Lancefield precipitin method (51). Isolation of the organisms and grouping by the precipitin method furnished the basic data against which all other methods were evaluated. When swabs were incubated in Todd-Hewitt Broth for 3 hr prior to preparation of pour plates, and the latter were examined for β-hemolytic colonies resembling group A streptococci after 18 hr of incubation, only 1 of 177 specimens positive for group A streptococci was missed. However, this method yielded a high incidence (9.6% of the total 1,115 cultures) of β-hemolytic streptococci that were not group A. None of the streak-plate techniques and only one of the FA procedures gave good results. The latter consisted of preparing pour plates, as described above, and transferring β-hemolytic colonies to broth for 3 hr of incubation, followed by preparation of smears and staining with the FA reagent. By use of this method, all 162 specimens that were positive by the precipitin test were currently identified, and only three false positive reactions due to cross-reactions were obtained. The authors concluded that the saving of at least 1 day in reporting the presence of group A streptococci in a throat specimen was of considerable importance. In their view, the FA procedure provided the most practical approach for the identification of these organisms and the subsequent treatment of infections caused by them.

Karakawa, Borman, and McFarland (34*a*) described the preparation of FA for type 1, group A streptococci. Staining reactions were determined to have resulted from a type-specific anti-M protein interaction. Typing of group A streptococci from epidemiological studies would be facilitated if similar reagents for the identification of the remaining 45 serological types could be developed.

A simple and quite satisfactory reagent for routine identification of group A streptococci by immunofluorescence consists of a mixture of labeled $(NH_4)_2SO_4$-fractionated antiglobulin and either group C *Streptococcus* antiserum or normal rabbit sera. The latter sera should be selected and tested to determine whether they contain antibody that reacts with groups C and G streptococci and with coagulase-positive staphylococci.

The magnitude of the routine use of the FA procedure for grouping of streptococci is convincing evidence of its practical value as a tool for more rapid and economical grouping of these organisms. An important by-product has been an increased awareness of the need for grouping human β-hemolytic streptococci to assist the clinician in the prevention and control of streptococcal infections. A significant error may result from the assumption that β-hemolytic streptococci from the human nasopharynx belong to group A. In some studies, up to 50% of such cultures belonged to groups not having the same clinical significance with respect to human disease as do group A streptococci (72).

Serogrouping of Enteropathogenic Escherichia coli

Although it has not yet received the widespread application it deserves, the FA test for detection of the enteropathogenic *E. coli* (EEC) offers a marvelous screening device for surveillance of institutional diarrhea of infants. The test, originally developed by Whitaker et al. (88), was surprisingly specific for the nine serogroups of EEC most commonly encountered as the etiological agents of infant diarrhea in the United States. Diagnostic conjugates may be prepared from good OB agglutinating sera by separating and labeling the γ-globulins. In our laboratory, these reagents were pooled as follows for the screening of fecal smears. Pool 1 contained labeled antibodies for *E. coli* O26:B6, O55:B5, O111:B4, and O127:B8; pool 2 contained antibodies for serogroups O86:B7, O119:B14, O125:B15, O126:B16, and O128:B12.

Methods for preparing reagents and for applying the test were given in detail by Thomason et al. (80). These reagents may be used unsorbed without fear of significant cross-reactions if the titer permits a dilution of 1:20 or more based on the original volume of globulin. These and other labeled OB antibody preparations for *E. coli* may be expected to give additional common antigen fluorescence if they are used at concentrations higher than necessary to obtain optimal staining. From a practical point of view, dilution is the simplest and most effective means of enhancing the specificity of conjugates.

False positive reactions usually are due to Enterobacteriaceae possessing antigens common to the EEC. Strains of *E. coli* and of *Citrobacter freundii* are the species most frequently isolated (Thomason, *unpublished data*). Serologically rough and untypable strains of *E. coli* constitute the largest cross-staining group. At times,

lanceolate-shaped enterococci staining brilliantly with the conjugates are encountered, but these are easily differentiated from *E. coli* by the experienced worker. If conjugates for *E. coli* O124:B17 are included in the reagents used for screening fecal specimens, false positive reactions, presumably due to common antigen fluorescence, may be obtained. This appears to be a problem primarily of commercial reagents, since the O124:B17 conjugates used in this laboratory have not proved troublesome. It should be remembered that the OB antigens of O112:B11 and O124:B17 serogroups are identical to those of *Shigella dysenteriae* 2 and *S. dysenteriae* 3, respectively. These and other labeled OB antibody preparations for *E. coli* should not be used at concentrations greater than their diagnostic dilutions. There is no indication that normal antibody against the EEC contributes to nonspecificity, at least insofar as specific pathogen-free rabbits are concerned. More data are needed on "serologically dirty" rabbits from commercial sources.

The examples discussed above are exceptional, since extensive experience has proved that it is unusual to encounter in fecal smears non-EEC bacteria morphologically and serologically resembling EEC and staining with the specific conjugates. Data compiled recently by Berenice Thomason in our laboratory, based on the study of almost 5,000 fecal specimens, show that only 5.3% of the FA-positive cultures failed to be confirmed as EEC. When serogrouping of isolates by immunofluorescence was compared with serogrouping by slide agglutination, the latter being a procedure that is not recommended but which is nevertheless widely employed, it was found that exactly twice as many false positive reactions were obtained by slide agglutination. All non-EEC, FA-positive cultures were also positive by slide agglutination.

The great value of the FA test for EEC lies in its use for screening negative specimens which require neither culturing nor testing of colonies by agglutination with the attendant cost of media and labor. Several additional studies have confirmed and extended the scope of the original work (3, 6, 7, 14, 54, 55, 58, 69, 80).

The FA procedure for detection of EEC generally has been found to be more sensitive than cultural methods. Three separate studies conducted by our laboratory, one in Puerto Rico by Cherry et al. (6) and two in Atlanta by Boris et al. (3) and Thomason (*unpublished data*), have shown that approximately one-third (32%, 35.7%, and 30.5%, respectively) of the total positive specimens (FA and culture) from cases of diarrhea in infants failed to yield the organism on culture. In the first study, involving 291 infants, every specimen was both cultured and stained by the FA reagents. No specimen was found to be positive by culture when negative by immunofluorescence. Since this and subsequent experience indicated that specimens culturally positive but FA negative were rarely encountered, the FA staining technique was used to screen for positive specimens which then were cultured. This procedure was followed in the last two studies mentioned above and emphasizes the importance to the epidemiologist of the FA test as a tool for rapid and economical screening of fecal or throat specimens for the presence of the EEC.

Evidence, mostly of an indirect nature, has indicated that the majority of the excess of positive FA over positive cultural results represents increased sensitivity of the former test rather than false positive reactions (6, 69, and Freid and Lepper, *unpublished data*). Some of the relevant points are as follows. (i) EEC can be isolated by repeated culturing of some specimens which were originally negative. (ii) Few heterologous staining reactions were encountered when the conjugates were tested against a variety of *Enterobacteriaceae*. (iii) Positive staining reactions were absent in adults both with and without diarrhea and rare in children without diarrhea unless the latter also were culturally positive. (iv) The culture method failed due to drug therapy, lytic bacteriophage, bacterial antagonisms, or other reasons.

An interesting by-product of the immunofluorescence studies of EEC has been the observation of the occurrence of asymptomatic nasopharyngeal carriers of EEC, both adults and children (3). During an epidemic period these were found more frequently than intestinal carriers among the families and close associates of infants hospitalized with diarrhea. The serogroup of *E. coli* detected usually corresponded to that recovered from the acute case. Direct transmission of EEC from asymptomatic individuals, carrying EEC in the nasopharynx but not in the stool, to susceptible members of a household by means of aerosols or by mouth contact was implied by the data obtained. This mechanism of infection is a distinct possibility requiring further investigation.

The availability of FA procedures and reagents for serogrouping of EEC should stimulate hospital and public health laboratories to undertake the diagnosis of *E. coli* diarrhea of infants. As pointed out by Warren Wheeler in a hard-hitting editorial (87), the failure to recognize and cope with in-

stitutional diarrhea due to the EEC is a national disgrace.

Detection of Treponema pallidum and Its Antibody

Fluorescent treponemal antibody (FTA) test. The FTA test has a limited but important application in syphilis serology. The test, as developed and modified by Deacon et al. (17, 19), is based on the ability of antibody from the serum of the patient to be specifically sorbed onto dried smears of *T. pallidum*. Fluorescein-labeled antihuman globulin is employed as an indicator of the primary antigen-antibody combination. This is then an indirect FA test in which the unknown component is the presence or absence of antibody in the patient's serum. The FTA test was included in the battery of serological tests for syphilis that were compared on a national basis under the sponsorship of the Venereal Disease Branch of the Communicable Disease Center in 1957. The result of this comparison showed that the FTA test possessed a specificity and sensitivity exceeding that of the other treponemal tests and equal to that of the nontreponemal tests which were in routine use (68). Several evaluations (2, 5, 13, 27, 37, 40, 52, 53, 56, 62, 70, 71, 76, 82, 91) have confirmed the earlier work of Deacon and his associates and have, in general, validated the specificity and sensitivity of the FTA test with sera from a variety of patients under a variety of test conditions. The procedure for performance of the FTA-200 test was included in "Serologic Tests for Syphilis" in 1964 (82*a*), and protocols for testing of the reagents employed in this test were published (73).

Most investigators agree that the FTA test is simpler, more rapid, and less expensive than the treponema immobilization (TPI) test, which explains the widespread replacement of the TPI test by the FTA test. Some investigators felt that differentiation of positive from negative results in borderline reactivity was more difficult with the FTA than with the TPI tests (5). There have been minor disagreements among workers concerning the specificity and sensitivity of the FTA test relative to that of the TPI test, and concerning the identity of the antibodies involved in the two tests (2, 13, 44, 75, 84, 91). Originally, it was thought that the two tests measured the same antibody, but recent information has indicated that this view may need to be modified (22, 33, 44).

As originally applied (17), a 1:5 dilution of the patient's serum was employed for the FTA test. Later, it was realized that this concentration of serum was giving too many false positive reactions, and the test was modified to permit the use of a 1:200 serum dilution (19). This resulted in increased specificity but lowered the sensitivity of the test beyond desirable limits in certain types of cases. Additional research showed that the lack of specificity of the FTA test at the higher serum concentrations was due to naturally occurring treponemal antibodies in human sera (22, 33). These represent antigens shared by the pathogenic and saprophytic treponemes, the latter having their habitat in the oral cavity or intestinal tract of man. By sorption of the interfering antibodies with sonic-treated materials of the Reiter treponeme, it was possible to use a 1:5 dilution of the patient's serum for the test. A careful evaluation of the new test, fluorescent treponemal antibody-absorbed (FTA-ABS), showed that its sensitivity was more than twice that of the FTA-200 test previously employed, and its specificity was equaled only by the TPI test (33). The sera evaluated included (i) a group from primary syphilis, (ii) a group from untreated, treated, and inadequately treated syphilis, (iii) sera from patients giving biological false positive reactions, and (iv) control sera from normal persons.

Harris et al. (30) compared the FTA test with other tests for syphilis on cerebrospinal fluids. They concluded that, if the FTA, TPI, and Kolmer Reiter Protein tests are measuring the same antibody in spinal fluids, the FTA test is the most sensitive. The amount of spinal fluid required for the FTA test also was less than that needed for any of the other three tests used. Another report showed that the FTA test performed with undiluted cerebrospinal fluid was more sensitive than the TPI test (53).

Vaisman et al. (83) adapted the FTA procedure to the detection of syphilitic antibody in blood taken by finger puncture and dried on blotting paper. The FTA, TPI, and lipoidal antigen tests were compared on eluates of blood preserved by drying on blotting-paper discs for periods of up to 60 days at 20 to 25 C. The results of these tests were compared with those derived from applying the same three tests to sera taken from the same individuals by venipuncture. With a serum dilution of 1:100, no significant variations were found in sensitivity, specificity, or reproducibility when the FTA test was performed on dried blood obtained by finger puncture as compared with serum obtained by venipuncture. In some cases of primary syphilis, it was observed that antibody demonstrable by the FTA test appeared in the serum earlier than did that detected by the TPI or lipoidal antigen tests. On the other hand, the sensitivity of the TPI test proved superior to that of the FTA technique in comparative examinations of old, treated

syphilitic infections. The simplicity of the dried-blood method appears to offer an important advantage in syphilis serology when it is necessary to collect blood in areas far removed from the laboratory or where venipuncture is impractical.

Immunofluorescence of T. pallidum. Edwards (24) employed the indirect FA staining procedure for the examination of slides prepared from the exudate of primary syphilitic lesions. When the immunofluoresence procedure was compared to conventional dark-field examinations, complete agreement on both positive and negative slides was obtained. The new test proved superior to dark-field examinations with respect to ease of detection of the treponemes and the rapidity with which they could be identified among the cellular debris. It was shown that unfixed prepared slides could be maintained under a variety of storage conditions for periods up to 1 week or more without impairing the staining characteristics of the treponemes. The value of preserving specimens in this manner for examination at a later time or place appears obvious.

The successful detection of *T. pallidum* in a mixture of treponemes such as may be encountered in chancre fluid is dependent upon the specificity of the staining reagents. Deacon and Hunter (22) studied the relationshipsexisting between various strains and species of *Treponema.* The presence of a common antigen in these cultures furnished an explanation for the modification of the FTA test that had been found necessary earlier (19). Antibody for other species of treponemes either must be rendered ineffective by dilution of the patient's serum prior to the test, or it must be removed by sorption with appropriate antigens. Fortunately, serum titers for the wild treponemes were found to be considerably lower than that for *T. pallidum* in an infected patient.

Yobs et al. (94) solved the problem of nonspecificity by sorption of their conjugates with Reiter treponeme antigen to remove the treponemal group antibody prior to using these conjugates for specific staining of *T. pallidum* in syphilitic lesions in both rabbit and human tissues. These workers used both direct and indirect FA staining methods with equal success. They did not find the use of rhodamine-labeled bovine serum-albumin as a counterstain to be helpful for reduction of background fluoresence but commented on the disappearance of background staining as conjugates were diluted.

Recently, Kellogg and Deacon (35) described a rapid immunofluorescent staining (RIS) procedure for the identification of *T. pallidum* and *Neisseria gonorrhoeae* in smears from chancres and from cases of gonorrhoeae, respectively. Specificity, sensitivity, and intensity of staining obtained in a procedure requiring less than 1 min of exposure to the conjugate was said to equal that given by the usual technique requiring approximately 1 hr to complete. The staining is accomplished by rapid drying of the labeled antibody onto the smear at 45 C followed by rapid rinsing. The RIS procedure deserves further study, with particular attention to its effect on contrast between specific and background fluorescence. It may prove generally valuable in immunofluorescence work.

Most public health laboratories employ the FTA test as a substitute for the TPI, since the two tests give approximately the same results, and because preserved suspensions of treponemes may be used for the former, whereas fresh living suspensions from the rabbit testicle are required for the latter. Some laboratories confirm all positive Venereal Disease Research Laboratory tests by performing the FTA test. We are aware of one state laboratory that is currently making 6,000 to 7,000 of these examinations annually.

Identification of N. gonorrhoeae

Progress in the development and use of immunofluorescence as a tool for the identification of the gonococcus in exudates from cases of the disease has been rapid since the initial report of Deacon et al. (18). The discovery of the existence in freshly isolated cultures of an antigen resembling the K antigens of *E. coli* or the Vi antigen of *S. typhosa* was the key to the design of a specific staining technique for *N. gonorrhoeae.* This antigen apparently is specific for the gonococcus and renders cultures, in which it is fully developed, inagglutinable in O sera. It is transitory in nature, being rapidly lost from cultures after isolation. This characteristic probably accounts for the fact that it has remained undiscovered until recent years and also for the confused state of knowledge that has existed regarding some aspects of the serology of the gonococcus. Deacon et al. (18) showed that conjugates prepared from sera produced by injection of K antigen-containing cells could be freed from cross-reactions by appropriate sorption with a group A strain of *N. meningitidis.* Further discussion of the development and use of the FA method for the identification of *N. gonorrhoeae* may be found in another paper by Deacon (21). Danielsson (15, 16) recently repeated and extended the observations first reported by Deacon regarding the preparation of suitable diagnostic reagents and the testing of these for specificity. He recommended sorption of gonococcus conjugates by strains of *Staphylococcus aureus* giving a strong common antigen fluorescence as a means of rendering the FA re-

agent specific, rather than sorption with *N. meningitidis*. Sorption with staphylococci was said to eliminate all troublesome cross-reactions with *Neisseria* species other than *N. meningitidis*, reactions with the latter being disregarded because it is rarely found in the urogenital tract. The validity of this assumption must await further study. Fluorescein-labeled antimeningococcus conjugates may aid in obtaining such data.

With minor modifications, reagents of the type described above have been used in field evaluation of the FA technique for the gonococcus by several service laboratories. In one study by Deacon et al. (20), cultural procedures were compared with both the direct and delayed FA techniques on specimens obtained from 50 women who were named contacts of men with gonorrhea. Specimens were obtained from three sites (urethra, cervix, and vagina) in each patient, and each specimen was examined by all three techniques. The delayed FA test was performed on heavy smears prepared from cultures on a suitable growth medium that had been incubated for 16 to 20 hr at 35 C.

It was shown that the delayed FA test and the cultural procedure gave positive results in the same percentage of patients, although the delayed FA test revealed more positive sites. Less than one-half as many patients were found to be positive by direct FA test as by either delayed FA or cultural procedures. When the direct and delayed FA tests only were used in the examination of another group of 100 women, similar results were obtained. The authors considered that positive findings by either of the two FA tests constituted a completed examination. They emphasized that the delayed FA test must be completed if the direct FA examination proves negative. Attention also was called to the saving of time and money inherent in the use of either one of these tests.

Harris et al. (31) successfully used the delayed FA technique to determine the incidence of *N. gonorrhoeae* in 213 imprisoned females, none of whom had clinical symptoms of infection with this organism. Surprisingly, they found 20.6% of this group to be positive when specimens from the cervix, urethra, and vagina were examined. As might be expected, increasing the number of sites examined or the number of examinations made resulted in more positive findings. They concluded that asymptomatic females may constitute a large source of infection that is important in the perpetuation of gonorrhea.

A subsequent study of gonorrhea in asymptomatic teen-age and young adult females was conducted under controlled conditions to evaluate further the delayed FA technique in comparison with Gram-stained smears and culture for diagnosis of gonorrhea (4). The findings were very similar to those reported above; approximately 20% of this group showed asymptomatic gonococcal infection. In all cases, the delayed FA test gave results equal to, or better than, those obtained by culture. In some patients, gonococci were detected in specimens from the anus, and the necessity of examining this site was emphasized.

The value of the FA test for detection of the gonococcus in the male also has been substantiated by Moore et al. (50). Urethral swabs from 477 patients were used to inoculate plates for isolation and to inoculate slants of an appropriate medium for performance of the delayed FA test. It was concluded that the latter was equal, if not superior, in sensitivity to carefully performed cultural examinations. The results of the two tests were in agreement in 92.4% of the cases. In 5.7% of the cases, the FA test was positive and the culture negative; in 1.9%, the reverse was found. It was pointed out that the delayed FA test is more time-consuming than a presumptive cultural examination (gram-negative diplococci that are oxidase-positive). However, the value of either the direct or delayed FA procedure was established when a rapid confirmation of a clinical diagnosis was required. The sensitivity of the new method suggested its usefulness in the diagnosis of nonspecific urethritis and complications of gonorrhea, as well as in the detection of possible asymptomatic male carriers of the organism. In addition, the FA procedure may be expected to assist in clarifying the problem of continued infectivity after treatment.

Using antigonococcus conjugates sorbed with an appropriate strain of *S. aureus*, Danielsson (15) compared the direct and delayed FA test with cultural examination and direct microscopy for the diagnosis of gonorrhea in both male and female patients. The results were in agreement with those previously reported; the FA test proved more sensitive than either of the other two methods. The delayed FA test gave a much greater number of positive results in both male and female patients than did the direct FA test.

Lind (42) reported that cultural and FA methods gave similar results when she applied these to the diagnosis of gonorrhea in both male and female patients. She called attention to the problem of unwanted staining reactions that occurred when staphylococci were present in the material undergoing examination, but she assumed that this problem could be eliminated by further study. Fluorescent leukocytes were troublesome, but it is not clear to what extent

this fluorescence may have been due to specific gonococcal antigen dispersed throughout the smear rather than to natural fluorescence of the white cells or uptake of unreacted fluorescent material from the conjugate.

The most recent application of immunofluorescence to the diagnosis of gonorrhea was that reported by Price (61). In this study, five service laboratories examined 88,000 specimens obtained from 9,900 females. Specimens collected from the cervix, urethra, and vagina were examined by use of the two FA methods described above, and by culture. Of the total number of females examined, 36% were positive at one or more sites with the delayed FA technique as compared with 22% positive with culture and 15% with the direct FA test. When females named as contacts of males with gonorrhea were examined, 63% of those without clinical evidence of gonorrhea and 94% of those with clinical evidence of gonorrhea were positive with the delayed FA test. Corresponding figures for conventional cultural examination were 48 and 68%, respectively. There was a great deal of variation in the relative efficiency of the cultural and FA procedures among participating laboratories. Price attributed this to a lack of uniformity in performing conventional cultural procedures, since previous investigators had obtained equivalent results with the cultural and delayed FA procedures.

The status of FA tests for gonorrhea may be summarized by stating that, if the recommendations of the authors of the test are followed (28, 73), immunofluorescence appears to be a highly specific and sensitive tool for the identification of *N. gonorrhoeae*. As such, it may be expected to become a significant factor in the diagnosis and control of an important public health problem. When time is a factor of importance, the FA test has an additional advantage, since results having a reliability approaching that obtained by isolation and characterization of the gonococcus by fermentation reactions may be obtained in less than 1 day with the FA test as compared with the 3 to 4 days required with the conventional method.

Detection of Corynebacterium diphtheriae

The possibility of using fluorescein-labeled antitoxin for specific identification of toxinogenic strains of *C. diphtheriae* was explored first by Jones and Moody (34). In these preliminary studies, they used commercial diphtheria horse antitoxin to stain both toxinogenic and atoxinogenic cultures of the organism. Most of the former were stained brilliantly, and the latter commonly gave moderate staining reactions. It was surmised that staining of the atoxinogenic strains was due to cellular-antibody content of the labeled antitoxin. In spite of problems related to cross-staining reactions with *S. aureus* and with β-hemolytic streptococci, Jones and Moody (34) felt that the labeled diphtheria antitoxin was of value for identifying toxin-producing *Corynebacterium* species.

Whitaker et al. (89) subsequently (1961) reported excellent results with fluorescein-labeled diphtheria horse antitoxin (Wyeth Laboratories, Philadelphia, Pa.) for the diagnosis of diphtheria by direct FA staining of pharyngeal swab specimens. In their series of 90 nasopharyngeal swabs, 9 were obtained from cases diagnosed clinically as diphtheria; 8 of the 9 were positive by FA and 6 by culture. Of 10 cases diagnosed as streptococcal pharyngitis, nine were negative by FA and 1 was positive. Thirty additional cases which had been diagnosed as diseases other than diphtheria and 50 healthy control infants were FA negative. The enthusiastic remarks of the authors regarding the specificity of labeled diphtheria antitoxin for demonstrating toxinogenic *C. diphtheriae* were not in accord with the findings of Moody and Jones and have not been confirmed in subsequent studies. In fact, Moody and Jones (47, 48) abandoned the use of labeled antitoxin in favor of antibacterial reagents. This was due largely to the inability of the labeled antitoxin to differentiate toxin- from nontoxin-producing strains with reliability. Even antitoxin prepared in rabbits with highly purified Pope toxin was subject to the same limitations (Moody, *unpublished data*). Allen and Cluff (1) investigated the specificity of labeled diphtheria antitoxin (Wyeth Laboratories and Massachusetts Department of Public Health Biological Labs) for cultures of *C. diphtheriae* and related organisms. Their study was very carefully planned and executed, and their cogent analysis of the results offer convincing evidence of the inherent lack of specificity of labeled antitoxin for differentiation of toxinogenic and atoxinogenic cultures, or even for specific detection of *C. diphtheriae*. They suggest the possibility that the somewhat better staining of toxin-producing strains by labeled antitoxin may be due to factors other than the presence of toxin at the cell surface.

The positive FA staining reactions obtained by Whitaker et al. (89) on nine clinically typical cases of diphtheria were not confirmed by culture in three cases. This is a rather high percentage of cultural failures, since the patients apparently were in the acute phase of the disease and since blood tellurite medium is quite selective for the diphtheria bacillus.

It seems quite clear that, unless methods can

be devised to eliminate cellular antibody for *C. diphtheria* and other bacteria from labeled diphtheria antitoxin, little diagnostic use will be made of this reagent for detection of toxin-producing strains of *C. diphtheriae*. The difficulties are compounded by the fact that antitoxin usually is prepared in horses whose sera contain a variety of normal antibody components.

The most promising approach for identification of *C. diphtheriae* by use of the FA procedure is that described by Moody and Jones (47, 48). Using as a basis for their work the serological study of Lautrop (39), they selected two OK strains of *C. diphtheriae* whose conjugates produced staining of all cultures of this organism that were tested and did not stain other species, with the exception of the closely related diphtheria toxin-producing *C. ulcerans*. The reagent did not differentiate toxinogenic and atoxinogenic strains. The authors commented on the cross-reactions encountered with streptococci and with *S. aureus* and discussed ways in which these reactions could be reduced or eliminated. After preliminary use of the FA reagents on clinical material, they concluded that the FA test for *C. diphtheriae* was as specific and as sensitive as conventional methods. Direct examination of throat-swab specimens was unsatisfactory, and it was necessary to incubate the swabs in broth for a few hours prior to making smears for FA staining.

Subsequent field evaluation by W. D. Jones, W. L. Jones, and M. D. Moody (*unpublished data*), and by other workers using their reagents, has yielded satisfactory results in three separate studies in which immunofluorescence and cultural techniques were compared. The number of patients in these three studies was 624. In the first trial, 63.6% of the specimens were positive by culture; in another, 15%; and in the third, 13%.

A fourth study (V. J. Lewis and W. L. Jones, *unpublished data*), encompassing 950 specimens from school children, showed poor correlation between the FA and cultural results. The key to the explanation for this discrepancy was thought to be the nature of the population sampled: in contrast to the three earlier studies, only 3.8% of the specimens were positive culturally, and clinical disease was not common. It was reasoned that the number of diphtheria bacilli carried in the nasopharynx of the children approached a critical sensitivity level for detection by either of the techniques used. Thus, by chance, one specimen was positive by FA only, a second positive by culture only, etc.

An additional reason for the poor correlation between results of the two tests in the fourth study was suggested by the observation that only 9 of the 36 culturally positive specimens were positive by the FA test. This result indicated that culture may have been somewhat more sensitive than FA under the technical conditions employed in the study, a suggestion later verified experimentally (V. J. Lewis and W. L. Jones, *unpublished data*). The FA reagent is known to cross-stain occasional strains of diphtheroids, and staphylococci may be troublesome in this way if present in large numbers.

In summary, labeled antibacterial conjugates are applicable to the detection of *C. diphtheriae* in suspected or known cases, i.e., to persons with sore throats or a recent clinical diagnosis of diphtheria, or their close associates. The value of the test lies in the rapid presumptive diagnosis of diphtheria, but it must be used in conjunction with cultural procedures. The specificity of the test may be improved by finding ways of producing sera of higher specific titer, thus minimizing the effect of normal antibody.

Identification of Bordetella pertussis

Donaldson and Whitaker (23) were apparently the first workers to give serious consideration to the diagnosis of pertussis with FA techniques. They prepared conjugate for *B. pertussis* from rabbit serum and used it for the staining of homologous and heterologous cultures, and for direct staining of smears made from pernasal swabs of patients. Their subjects consisted of children showing typical clinical symptoms of whooping cough, patients with undifferentiated upper respiratory infection, and patients without respiratory-tract disease. The following groups of patients were positive with the FA test: most of 36 patients with clinical pertussis that had not been treated; all (13) of those that had been treated less than 48 hr; 2 of 16 that had been treated more than 48 hr. Of 10 asymptomatic children exposed to whooping cough, 2 yielded positive tests, and none of the 36 normal controls was positive when first examined. However, two members of this group later became positive and both developed clinically typical pertussis.

Although subsequent parallel cultural and FA evaluation reported by other workers indicated that the data presented by Donaldson and Whitaker (23) were valid, it is unfortunate that the latter workers did not attempt to isolate *B. pertussis* and confirm its identity by conventional means.

Kendrick et al. (36) conducted a controlled comparison of cultural and immunofluorescence techniques for detection of the whooping cough bacillus. Pernasal swabs, consisting of cotton affixed to flexible bronze trolling wire, were used to collect exudate. These were incubated in a small amount of a liquid medium for periods not

exceeding 2 hr, because the bronze wire is toxic to *B. pertussis* if contact is maintained for longer periods of time. In an examination of 130 children, the cultural and FA methods yielded corresponding results, either positive or negative, in 119 cases. Four specimens were culturally positive and FA negative; 7 were culturally negative and FA positive. The data suggested that cultural failure in the latter could be due to (i) inhibitory effect of bronze wire, (ii) prior therapy, (iii) nonviability of the organisms late in the course of the disease, (iv) nonspecific staining, or some combination of these factors. The authors were most concerned with the fourth possibility and showed that a certain amount of cross-staining of *B. bronchiseptica* occurred as well as staining of staphylococci and an occasional strain of a small coccus. They were unable to prepare sorbed sera of suitable specificity for *B. pertussis* due to an unexplained inability of factor sera to stain the homologous organisms. They concluded that the FA procedure offered real promise as an aid in the identification of *B. pertussis* but urged further evaluation. Later, it was noted that no FA cross-reactions occurred between *B. pertussis* and *B. parapertussis*, so that conjugates for both should be used in diagnosis to detect the presence of the latter in diseases resembling pertussis (25).

Holwerda and Eldering (32) employed the FA procedure in the direct staining of growth from Bordet-Gengou plates inoculated with pernasal swabs from 517 suspected cases of whooping cough. With this combination of culture and of FA staining of growth at the first sign of its appearance on the plates, they were able to diagnose *B. pertussis* 1 day earlier. Of the number of specimens positive by either or both methods, identical percentages (13.3) were positive by one method and negative by the other and vice versa. Conjugates processed from *B. pertussis* antiserum made in chickens gave higher specific titers, and cross-staining of staphylococci was eliminated.

Recently, in France, Marie et al. (43) reported obtaining more positive results with FA than with culture among their series of pertussis cases. An apparently false positive reaction was obtained on a specimen from 1 of 45 patients who had no clinical symptoms of pertussis.

It appears that the FA procedure may become a valuable tool for detection of the whooping cough bacillus either by direct staining of material removed from pernasal swabs or by staining of young cultures from isolation media. Further study is needed to find the best means of preparing specific conjugates and to develop less toxic swabs and transport media for collection and preservation of specimens prior to laboratory examination.

Identification of S. typhosa

Several years ago, it was shown that immunofluorescence could be used with great precision to localize and identify the antigens of typhoid bacilli and other salmonellae (78). In pure culture, the specificity of staining of *S. typhosa* and related bacteria with FA was roughly equivalent to that shown with agglutination tests. This led to an attempt to use fluorescein-labeled conjugates derived from polyvalent *Salmonella* O sera for specific detection of this genus in fecal smears (79). Conjugates prepared from O sera of *S. typhosa* and *Vibrio comma* and those from normal rabbit globulin were evaluated concurrently. The results were disappointing in that cross-serological reactivity was a serious problem, as shown both by the results of studying pure cultures of the various Enterobacteriaceae and by studies of fecal specimens from typhoid carriers, patients with gastroenteritis, and normal persons. For example, stained organisms resembling the typhoid bacillus were observed in 88% of the fecal specimens from 25 normal persons when smears were stained with the *Salmonella* polyvalent conjugate; 68% were stained with the typhoid O conjugate, and 84% by the cholera conjugate. It was concluded that this approach to *Salmonella* identification was impractical, insofar as its application to the examination of feces was concerned.

The unexpected specificity of immunofluorescence for the detection of the enteropathogenic *E. coli* as discussed earlier led to a re-examination of the possibility of using this technique for the screening of fecal specimens from typhoid carriers. The success achieved has been due to recognition of the fact that the specificity of the FA technique for *E. coli* is dependent, to a large extent, on the use of conjugates containing relatively large amounts of K(B) antibody. Likewise, the Vi antigen of *S. typhosa* is a K antigen; although it is found in other *Salmonella* and in non-*Salmonella*, it is apparently uncommon. Therefore, in contrast to labeled O antibody, labeled Vi globulin has proved to be a highly specific reagent for detection of the typhoid bacillus. Recently, fecal smears from 130 persons previously proven bacteriologically to be typhoid carriers were examined concurrently, but independently, with FA and conventional cultural techniques (81). Two fluorescein-labeled antiglobulins were used; one was Vi antibody purified by sorption, and the other was unsorbed O,Vi antibody for *S. typhosa*. Of the 130 carriers, 89 (68.5%) were positive for *S. typhosa* with FA, whereas 90 (69.2%) were positive with culture. Control specimens were negative with both tests. The

fluorescein-labeled Vi antibody gave more positive results than did the O,Vi reagent. There were no significant differences in the two techniques with respect to either sensitivity or specificity. The combined use of cultural and FA techniques permitted detection of more carriers than either method alone. The new laboratory procedure may be expected to expedite the diagnosis of acute typhoid fever and aid in the detection of typhoid carriers, both in connection with outbreaks of disease and in the screening of immigrants in quarantine stations.

The success of the FA technique for the detection of a specific *Salmonella* species suggests the desirability of further studies to determine whether *Salmonella* of other serotypes possess K antigens that could enhance the specificity of their reactions with the appropriately labeled antibody.

Grouping of Shigella

Most of the published work dealing with the application of immunofluorescence to the recognition of *Shigella* has appeared in the Russian literature, and the results often are difficult to interpret due to lack of technical details.

LaBrec et al. (38) explored the possibility of using both labeled grouping and typing sera for detection of *Shigella* in pure culture, in fecal smears of both normal and experimentally infected guinea pigs, and in normal fecal specimens from man. The direct FA staining procedure gave results which had the same degree of specificity as slide agglutination tests when either group- or type-specific reagents were used on pure cultures. Attempts to detect *S. flexneri* in fecal specimens of experimentally infected guinea pigs were successful if type-specific FA reagents were used, but group-specific reagents gave cross-reactions that were troublesome, except in the presence of large numbers of *Shigella*. Fecal smears from normal human volunteers gave nonspecific staining with grouping conjugates but not with typing conjugates.

Cherry et al. (6) used a conjugate for *S. sonnei* for detection of that organism in fecal specimens from 291 Puerto Rican children mostly under the age of 2 years. The conjugate was prepared from serum containing antibodies for both antigen forms of *S. sonnei*. The specimens were cultured simultaneously and 12 specimens yielded fluorescing organisms resembling *Shigella*. *S. sonnei* was isolated from 10 of these, and from 6 additional specimens that were negative with FA staining. Taylor et al. (74) compared cultural methods with immunofluorescence for the detection of infections due to *S. sonnei*. The two methods were in agreement in 73% of the 394 specimens examined. In 57, or 14.5% of the specimens, positive results were obtained by culture only; in 52, or 13.2%, positive immunofluorescent staining was not confirmed by culture. The cultural techniques used were carefully worked out and pretested to insure maximal recovery of *S. sonnei* from fecal specimens. In this study, the use of a highly selective medium, such as deoxycholate-citrate agar, apparently rendered the FA test less sensitive than cultural methods. The authors attempted to determine the cause of the positive FA staining results which could not be confirmed culturally. They offer convincing evidence that this staining was not due to nonviable *S. sonnei* organisms, because it occurred just as frequently in specimens from patients having no known contact with dysentery cases as it did in contacts and convalescent cases of *S. sonnei* infection. However, they were unable to isolate from the feces any other organism that could explain the positive FA reactions obtained. Nevertheless, sorption of the specific conjugate by the homologous form 1 and form 2 antigens of *S. sonnei* eliminated staining in the culturally negative specimens, indicating the presence of nonviable or noncultivable organisms having antigens in common with *S. sonnei*.

Subsequent evaluation of FA tests for detection of *S. sonnei* were reported by Taylor and Heimer (74*a*) after improvements in the making and reading of microscopic preparations. The FA and cultural methods agreed in 95.6% of the 388 specimens examined, 1.8% of the remaining specimens being FA positive but culturally negative, and 2.6% being positive by culture only. On the basis of this favorable evaluation, the authors now report, by telephone, positive FA tests on specimens from cases of acute diarrhea. This report usually can be made within an hour, but it is emphasized that it should be regarded as provisional, subject to confirmation by culture. This procedure appears to offer rapid and reliable presumptive diagnoses that can be of real assistance to the physician in areas such as Great Britain where the incidence of dysentery due to *S. sonnei* is quite high.

There are several difficulties inherent in devising a procedure for FA staining of *Shigella* in fecal specimens received in Public Health Laboratories. It is not practical to prepare sorbed conjugates for each *Shigella* serotype, although the work of LaBrec et al. (38) indicated that highly specific reagents could be obtained in this way. The requirements of the public health laboratory for FA reagents for *Shigella* can be met by the preparation of conjugates for the four serogroups (A, B, C, and D). Such reagents should be usable

at fairly high titer, require little (if any) sorption, and be highly specific for *Shigella*.

Current status and future prospects. Unpublished work from our laboratory indicates that grouping reagents with increased specificity can be prepared from sera made with cultures having well-developed K antigens. The specificity of the K antigens makes it necessary to include labeled antibody for most of the serotypes of *Shigella*, if complete coverage is desired. However, in a given geographic location, the detection of a high percentage of *Shigella* that are likely to occur may be achieved by pooling conjugates for a moderate number of serotypes into a group of four reagents representing the serogroups A, B, C, and D. A major problem is that of obtaining immunizing strains with well-developed K antigens. Defining conditions that will enhance production of this antigen in shigellae may prove to be the key to the development of a suitable FA technique. This approach is based on the premise that *Shigella*, in vivo, contain well-developed K antigens. Sera containing predominantly K antibody are necessary to minimize the effect of cross-reactions due to common O antigen relationships between shigellae and other Enterobacteriaceae. Conjugates prepared from sera possessing good K titers may be rendered more specific by dilution to reduce the participation of labeled O antibody in the staining reaction. Further development and evaluation are needed to determine whether shigellae in clinical specimens can be grouped reliably by the FA staining procedure. At present, it is not a practical tool except, perhaps, for detection of the highly specific antigens of *S. sonnei*. The nature of the clinical specimens to which the test is applied undoubtedly will be an important factor, since specimens from acute dysentery seem to constitute the most desirable test material.

Bacterial Meningitis

Page et al. (57) described the application of fluorescein-labeled conjugates to the identification of *Haemophilus influenzae* in spinal-fluid sediments. They examined 53 spinal-fluid specimens, 50 from cases of acute meningitis and 3 from convalescent cases. In conjunction with the FA procedure, they employed Gram staining and cultural examination. Positive or negative results were in agreement with all three methods in 48 of the specimens examined; in the remaining 5, fluorescent organisms were observed in the absence of growth on culture media. However, in all of the latter, there was clinical evidence consistent with a diagnosis of meningitis due to *H. influenzae* that had been successfully treated with antibiotics. Smears from spinal fluids of patients with meningitis proven to be due to other bacterial species gave negative results with the conjugate for *H. influenzae*. It was concluded that immunofluorescence in conjunction with conventional techniques offered a valuable tool for the rapid identification of etiological agents of bacterial meningitis.

Sell et al. (66) used conjugates for *H. influenzae* of types a, b, c, d, e, and f to study the staining reactions of a group of 73 recently isolated strains of this species. It was found that 31 cultures could be typed specifically by immunofluorescence, and only 16 were typable by agglutination tests. Indirect FA methods employing labeled antirabbit sheep globulin were not type-specific. Sell et al. (67) applied the FA inhibition test to the detection of antibody to the six serotypes of *H. influenzae* in the acute and convalescent serum of 39 children with acute upper respiratory infection. Strains typable by direct FA staining were isolated from 18 patients, all of whom developed antibodies that could specifically inhibit the fluorescence of the homologous strain. Paired sera were obtained from 19 of the 21 children yielding untypable strains of *H. influenzae*. Sera from 12 of these individuals contained type-specific antibody that inhibited the fluorescence of one of the six type-specific stock strains with which they were tested. Agglutinating antibody for the homologous strain only (not one of the six types) was detected in the serum of four children, and no fluorescence-inhibiting or agglutinating antibody was found in the serum of three children. It was surmised that the four children with agglutinating antibody for *H. influenzae* strains not belonging to types a, b, c, d, e, or f may have experienced infection with additional types of this species.

Eveland (26) correctly identified *Listeria monocytogenes* in the spinal fluid obtained from a patient with meningitis by staining of the sediment with a specific conjugate for this organism.

Although they did not apply their conjugates and techniques to clinical diagnostic problems, Metzger and Smith (43*e*) developed reagents for the identification of *Neisseria meningitidis*. Conjugates representing each of the four serological groups of meningococci and, in addition, two polyvalent reagents were tested on homologous and heterologous *Neisseria* and on a variety of other gram-negative and gram-positive bacteria. Intragroup cross-reactions among the cultures of *N. meningitidis* were encountered frequently, but cross-reactions beyond this species were confined to one strain of *N. gonorrhoeae*. The authors were impressed with the potential value of immunofluorescence for the rapid and specific diagnosis of acute meningitis and other infections caused by meningococci, pneumococci, and *H. influenzae*.

A recent investigation of the use of immunofluorescence techniques for identification of meningococci (29) and of *H. influenzae* in cerebrospinal-fluid sediments requires comment. Conjugates prepared from polyvalent (A, B, C, and D) meningococcal antisera and from *H. influenzae* type B antisera were said to be of excellent potency and specificity. The major cross-reactions given by the former were with *N. gonorrhoeae* and *N. catarrhalis*, neither of which would be important in spinal fluids. The results of cultural examination of cerebrospinal-fluid sediments from cases of both septic and aseptic meningitis were compared to Gram- and FA-staining reactions on the same specimens. Neither of the staining procedures was as sensitive as culturing for detection of meningococci or *H. influenzae*. The FA-staining procedure was comparable to the Gram stain for detection of meningococci, but it was inferior to the latter stain for the identification of *H. influenzae*. The authors were disappointed in the results that they obtained in the examination of clinical specimens by the FA technique. Their pessimism is open to question, since they offered no data in support of the use of absolute ethyl alcohol and freezing as a means of storage and preservation of cerebrospinal-fluid smears. This method of handling smears prior to staining may have been deleterious to the FA reactions. In addition, they failed to specify the filter combination used for observation of fluorescence. Appropriately matched exciter and barrier filter combinations are essential for obtaining good results, particularly when background fluorescence is a problem as it appeared to be in this study. Page et al. (57) obtained a much closer correlation between FA staining and cultural results for *H. influenzae* than was found by the above workers.

Studies in our laboratory during the past 2 years have shown that conjugate pools can be used to give rapid and specific identification of the common bacterial incitants of meningitis by direct staining of spinal-fluid sediments (Biegeleisen, *unpublished data*). In the large group of cases of meningitis that was studied, the FA and cultural results were in close agreement. Spinal-fluid sediments were stained with conjugates for *H. influenzae*, *N. meningitidis*, and *Diplococcus pneumoniae* and proved very reliable for the identification of these organisms.

Immunofluorescence is a highly sensitive technique for this purpose and, in addition, has the virtue of permitting detection of drug-inhibited or dead bacterial cells. By judicious selection of the species and serotype antibodies included in the conjugate pools, reagents can be prepared which will give adequate coverage of a very high percentage of all cases of bacterial meningitis without becoming too numerous or unmanageable. A specific diagnostic result obtained within 2 hr or less after the lumbar puncture serves to guide the clinician in making the most effective choice of a therapeutic agent. Dependence upon Gram staining or other nonspecific staining procedures leads to numerous errors, since these methods do not have the dimensions of serological specificity. Furthermore, the effects of the normal humoral and cellular defense mechanisms often render bacteria in spinal fluids atypical both in morphology and staining characteristics. There are numerous examples of this, e.g., mistaking *Herellea* for meningococci or *Listeria* for *H. influenzae*. The consequences of such mistakes may be disastrous for the patient, since the most effective drug or antibiotic may be entirely different in situations such as those just mentioned.

Miscellaneous Applications

FA methods have been used to advantage in the study and diagnosis of certain important but uncommon bacterial diseases. The types of specimens in which these organisms can be demonstrated successfully by immunofluorescence are shown in Table 4. These applications are discussed in some detail in the following sections.

Brucella. Conjugates suitable for the detection of antigens of *B. abortus*, *B. suis*, or *B. melitensis* may be prepared from antisera for any one of the three species, since these reagents cross-react strongly (47*a*, 50*b*, 64*b*). The pathogenesis of experimental *B. suis* infection in guinea pigs has been studied with the technique of immunofluorescence (50*b*). Both cellular and diffuse antigen was identified in acetone-fixed sections of frozen tissues by applying a conjugate for *B. abortus*. Accumulations of antigen and specifically stained cells of *Brucella* also have been demonstrated intracellularly in tissue-culture preparations of guinea pig leukocytes (33*b*). *Brucella* cells have been stained in the blood and in impression smears of the liver and spleen of experimentally infected guinea pigs and rabbits, and in liver, spleen, lymph nodes, and bone abscesses of naturally infected swine that were being processed in a commercial abbatoir (1*c*). In the swine, masses of specifically stained but diffuse *Brucella* antigen were found more commonly than were intact bacterial cells.

Scarborough (64*b*) compared the efficiency of FA and cultural procedures for the identification of *Brucella* in the blood of guinea pigs experimentally infected with *B. suis* and in the blood of patients with clinical brucellosis. In both guinea pigs and man, agreement was poor between the results of direct FA examination and culture, the

TABLE 4. *Detection of bacterial antigens and antibodies in various types of specimens by immunofluorescence*

Conjugates	Antigen in						Antibody in serum or tissue
	Culture	Body fluids	Tissue imprints	Formalin-fixed paraffin-embedded tissues	Frozen tissues	Tissue-culture cells	
Brucella	+ (1*c*, 47*a*)*	+ (1*d*, 64*b*)	+ (1*c*)		+ (50*b*)	+ (33*b*)	+ (1*d*, 47*a*)
Bacillus anthracis	+ (1*e*, 5*b*)	+ (1*e*, 41)	+ (1*b*, 1*e*, 5*b*, 27*a*)	+ (5*b*)			+ (5*b*)
Pasteurella pestis	+ (91*a*)	+ (4*a*, 46*b*, 91*a*)	+ (4*a*, 32*a*, 34*b*, 46*b*, 85*a*, 91*a*)		+ (70*c*, 85*a*)	+ (4*a*)	
Francisella tularensis	+ (33*a*, 43*a*, 43*c*, 46*a*, 64*a*, 93*a*)	+ (43*b*)	+ (43*a*, 46*a*, 64*a*)	+ (43*b*)	+ (43*b*, 89*a*, 89*b*)	+ (43*d*, 64*a*)	+ (43*b*)
Leptospira	+ (3*a*, 6*a*, 50*a*)	+ (3*a*, 6*a*, 89*c*, 89*d*)	+ (3*a*, 89*c*, 89*d*)	+ (42*a*)	+ (6*a*, 42*b*, 50*a*, 89*c*)		
Listeria monocytogenes	+ (1*f*, 26, 70*a*)	+ (1*f*, 26, 43*f*)	+ (1*f*)	+ (1*a*, 84*a*, 70*b*)			

* Numbers in parentheses refer to Literature Cited.

latter being from 30 to 56% more sensitive. However, when either heparinized or untreated blood was incubated in broth, complete agreement was obtained between the two techniques. Since detection by immunofluorescene was specific for smooth strains, identification of the genus *Brucella* could be made 4 to 10 days earlier than was possible by culture. Heparinization of the blood specimen permitted detection of *Brucella* approximately 3 days earlier than was feasible with untreated blood. Scarborough attributed the lack of sensitivity of the direct FA examination to (i) autoinhibition of the staining reaction by serum antibody and (ii) the inadequacy of methods for concentrating the organisms in blood.

The relative sensitivity of the agglutination test and the inhibition and indirect FA tests for assay of serum antibody was determined (64*b*). In contrast to the reports of earlier workers (1*d*), it was found that the indirect FA test usually gave titers only one or two tubes lower than the agglutination test, and the inhibition of specific staining of *Brucella* cells by sera having significant agglutination titers was sharply reduced when these sera were diluted as little as 1:5. Biegeleisen et al. (1*d*) compared the indirect FA test to the agglutination test for assay of *Brucella* antibody in human serum. The latter procedure consistently gave titers 5 to 20 times higher than those obtained by the indirect FA test. In an earlier paper (47*a*), it was indicated that the FA inhibition test and the agglutination test gave similar serum titers, the lowest titers being obtained by the indirect FA test. Differences in strains of *Brucella* used as antigen and differences in the reading of end points may partially explain these discrepancies, but further clarification is needed. In any event, except for the possibility that antibody(s) involved in immunofluorescence may be different from those which operate in agglutination tests (64*b*), the former procedure appears to have no advantages over the latter for the measurement of serum antibody.

The direct FA staining procedure for *Brucella* in culture or in tissue is both sensitive and rapid, and it undoubtedly will be used to advantage by veterinary pathologists and others interested in diagnostic problems or in the study of host-parasite interactions. A summary of these applications is presented in Table 4.

Pasteurella pestis. Winter and Moody (91*a*) were the first to employ FA techniques for the staining of *Pasteurella pestis* (Table 4). They produced sera both from whole cells and from the fraction I antigen of virulent strains and found these to be completely specific for *P. pestis* by using both agglutination and FA-staining tests. If care was used in the selection of the strain and in the preparation of the immunizing antigen, it was unnecessary to sorb the sera or conjugates to prevent cross-reactions with *P. pseudotuberculosis*. Conjugates prepared as described above stained all virulent and protective-avirulent strains; nonprotective avirulent strains were stained very slightly, if at all, reflecting the strong relationship between the presence of fraction I capsular antigen and the brilliance of immunofluorescence. One strain from a fatal human case of plague was discovered to be aberrant in that, although it possessed fraction I antigen as determined by gel-diffusion analysis, it was not stained by conjugates containing antibody for this antigen. Apparently, in this strain, the fraction I antigen either is located intracellularly or its reactivity with antibody at the surface of the cell is blocked in an unusual way. It was noted that fixation of smears of *P. pestis* by heat or by dioxane was superior to methanol, ethyl alcohol, or formalin fixation.

Moody and Winter (46*b*) demonstrated that FA reagents for *P. pestis* could be used successfully to stain plague bacilli in impression smears of experimentally infected mice. A strong presumptive diagnosis of plague could be obtained at least 2 days earlier than was possible if the organism was isolated and identified by conventional means.

Cavanaugh and Randall (4*a*) employed FA procedures in the elucidation of the role of *P. pestis* in flea-borne plague. Using methanol-fixed smears and the indirect FA test, they demonstrated that the well - encapsulated, phagocytosis - resistant cells, which also were rich in fraction I antigen and were stained brilliantly by the FA conjugate, corresponded to the highly virulent form of *P. pestis*. Immunofluorescence proved to be a useful tool for characterizing the antigenic state of the plague bacillus in the stomach of the flea and its fate after ingestion by phagocytic cells.

Kartman (34*b*) called attention to the value of fluorescein-labeled antiglobulin for detection of plague bacilli in the tissues of rodents found dead in the field. These conditions were unfavorable for the cultural recovery of the plague bacillus, but frequently permitted its demonstration by FA staining in smears of spleen or bone marrow. Hudson, Quan, and Kartman (32*a*) studied the efficacy of FA tests for detection of *P. pestis* in carcasses of infected laboratory mice stored for various periods of time at different temperatures. They concluded that the FA test was superior to animal inoculation or cultural methods for detection of organisms in mouse carcasses stored for 4 days or longer at 37 C, for 8 days or longer at 20 to 25 C, and for 34 days or longer at 4 to 5 C. In culturally negative tissues stored for 16 to 82 days

at 37 and 20 to 25 C, precipitin and FA tests had approximately equal sensitivity for detection of plague antigen. The FA test was somewhat more sensitive when tissue was examined which had been stored for longer periods at 4 to 5 C. Most striking was the demonstration by the FA test, but not by the precipitin test, of plague antigen in two guinea pigs whose tissues had been stored at room temperature for 7 years. Walker (85*a*) found immunofluorescence a useful tool for following the distribution and persistence of fraction I antigen of the plague bacillus in the tissues of guinea pigs under a variety of conditions. He demonstrated the antigen both in tissue-impression smears fixed in cold absolute methanol and in freeze-dried sections embedded and cut in paraffin. The paraffin was removed from the latter by the use of toluene, and the sections were fixed as were the impression smears. In this connection, it has been reported that xylene denatures the surface antigen of *P. pestis* when it is used to remove paraffin from sections, but that the specific antigen remains intact in frozen sections of formalin-fixed material (70*c*).

Three opportunities have arisen for examination of pathological material from fatal human cases of plague (Moody, *unpublished data*). In the first, *P. pestis* cells and diffuse antigen was specifically demonstrated in smears of aspirated bubo fluid, and cells were stained in smears from a macerated blood clot. In the second case, the fluorescent organisms were seen both in impression smears of frozen, unfixed lymph node and in formalin-fixed, paraffin-embedded sections of the lymph node. Methanol and formalin-fixed tissues from case 1 and formalin-fixed tissues from case 3 gave negative or inconclusive results. It is not clear whether formalin fixation or the use of organic solvents for paraffin removal are primarily responsible for denaturation of the fraction I antigen.

FA staining is the method of choice for the rapid and specific presumptive identification of the plague bacillus in tissues or in culture. Its effective use presupposes the availability of appropriate reagents and of a keen diagnostician who is aware of the possibility of plague infection. FA reagents also are the method of choice for the examination of animal carcasses, since the diagnosis may be made 2 days earlier than is possible by culture, or may be made rapidly and specifically in the absence of viable organisms. These investigations are summarized in Table 4.

Francisella tularensis. Globulin portions of highly specific antisera for *F. tularensis* (*P. tularensis*) were labeled with fluorescein isocyanate or isothiocyanate and used as FA reagents for identification of organisms in cultures and in tissues from infected animals (Moody, *unpublished data*, and 43*c*). Twenty strains of this species, varying from avirulence to full virulence for mice, were stained without difficulty. Either the direct or indirect FA procedure was applicable. Staining reactions could be inhibited by tularemia antiserum from rabbits or man but not by normal serum or by heterologous antisera. Either viable or formalinized antigens could be used to demonstrate the staining reactions. Most strains did not stain well when the organisms were heated for 2 hr at 100 C. Neither *F. novicida*, which is antigenically related, nor *Brucella* organisms reacted with labeled *F. tularensis* antiglobulin at diagnostic dilutions.

However, it was reported that avian antibody, having a high titer for *F. tularensis* by both agglutination and FA tests, cross-reacted strongly with a *Pseudomonas* sp. and with *Erysipelothrix insidiosa* (93*a*). The specificity of the conjugate was improved by appropriate sorption. It was stated that roosters immunized with living *F. tularensis* organisms produced antisera of higher titers and specificity than that obtained from monkeys or rabbits. In the opinion of the reviewers, more critical data are needed to support this statement.

Subsequently, Jaeger et al. (33a) used a high-titered (1:64) avian conjugate for detection of air-borne tularemia organisms. Living cells were collected on slides placed in Cascade impactors and killed by a variety of methods, all of which gave satisfactory preparations for FA staining. It was necessary to sorb the conjugate with streptococci to remove troublesome cross-reactions. The experiments indicated that fewer than 10 viable tularemia organisms per slide could be detected, with a high degree of specificity, by this method.

Earlier studies of the sensitivity of the FA test had shown that cells of *F. tularensis* could be detected with FA tests in smears made from suspensions containing fewer than 300 organisms per ml (46*a*).

By injection of cell suspensions of pure cultures of the organism, it was shown that the diagnosis of tularemia in mice could be made 48 to 72 hr earlier by employing FA tests than by conventional culture and agglutination techniques (46*a*). These data were derived from experiments in which impression smears of the tissues of mice injected with varying numbers of virulent *F. tularensis* cells were stained with homologous conjugate. At the same time, these inocula from the tissues were plated on appropriate media. The number of positive reactions obtained by the two methods was in close agreement, but the results of the FA tests were available soon after sacri-

ficing the animals, whereas the data from cultural and agglutination tests were not obtained for at least 48 to 72 hr. Regardless of the method used, the time required to make a diagnosis was generally inversely proportional to the size of the inoculum.

The above observations led to the use of mice as a menstruum for rapid propagation of *F. tularensis* from specimens of suspected cases of animal and human tularemia. In conjunction with cultural isolation techniques, mice were inoculated intraperitoneally with a saline extract of the macerated tissue specimen or with a culture. After 24 to 48 hr, representative mice were sacrificed, and duplicate smears were made from the cut surfaces of organs such as the liver, spleen, or heart. For FA staining, one smear was stained with a mixture of fluorescein-labeled *F. tularensis* antiglobulin and normal rabbit serum. The duplicate smear was stained with a mixture of same conjugate and *F. tularensis* antiserum. If the specimen was positive for *F. tularensis*, characteristic, highly fluorescent "blotches" of organisms were seen in tissue impressions, or well-formed fluorescent bacteria were visible in cultures. In duplicate smear, the fluorescence was inhibited by virtue of the presence of specific tularemia antiserum. This procedure was found to be extremely useful in a study of naturally infected rabbits (43*a*). Several opportunities to use the combination of mouse inoculation and the fluorescence inhibition test for rapid identification of *F. tularensis* have confirmed the validity of the test (Moody, *unpublished data*).

Riggs et al. (64*a*) employed the direct method to demonstrate FA staining of *F. tularensis* in heat-fixed smears of pure cultures and in acetone-fixed preparations from the tissues of infected mice and from cultured mouse fibroblasts (L cells). Other workers (43*d*) have followed the intra- and extracellular multiplication of this bacterium in the L strain of mouse fibroblasts, in bovine kidney cells, and in human amnion cells. Cover-slip preparations were washed in Hanks' solution, fixed for 10 min in cold acetone, and stained with a conjugate for *F. tularensis*.

The detection of *F. tularensis* in tissue sections from *Macacus* monkeys infected with the organism has been reported. In sections of formalin-fixed, frozen, and paraffin-embedded tissues, the organisms were identified and localized in the lung, spleen, and liver (89*a*). Intracellular forms of the pathogen were seen in macrophages of the lung and in Küpfer cells from the liver. In other studies (43*b*, 89*b*), the effect of tularemia vaccine in monkeys was followed when administered both aerogenically and dermally. Antigen was detected readily by immunofluorescence in acetone-fixed frozen tissue sections. To test for antibody in the tissues, frozen sections fixed with ethyl alcohol were exposed to tularemia antigen followed by fluorescent tularemia antiglobulin. Tularemia antibody was found to be localized in the lymph nodes and the spleen.

The indirect FA reaction has given reliable results in titrations of tularemia antibody either in animal or human serum. Although the titer obtained with this reaction was slightly lower than with agglutination tests, its specificity appeared to be of the same order (Moody, *unpublished data*).

Bacillus anthracis. Although Levina (41) apparently was the first worker to stain *B. anthracis* with fluorescein-labeled globulins, Cherry and Freeman (5*b*) described, in some detail, the preparation and use of such reagents. They demonstrated the usefulness of these conjugates in the detection of *B. anthracis* in culture, and in impression smears and sections of both human and murine tissue (Table 4). The stability of the capsular polypeptide, which enabled it to undergo formalin fixation followed by paraffin embedding and the usual histopathological preparative procedures without denaturation, was a surprising finding. Using conjugates made from sera prepared by injection of whole encapsulated cells of *B. anthracis*, they easily demonstrated the organism in sections of human tissue from three fatal cases of inhalation anthrax. By means of the indirect FA procedure, serological evidence was obtained which suggested that one patient represented a rare case of recovery from inhalation anthrax. Immunofluorescence was shown to be considerably more sensitive for detection of the organisms in tissue sections than the Brown and Brenn or the hematoxylin-eosin procedure. Subsequently, *B. anthracis* was demonstrated in sections of tissue from two additional fatal cases of human anthrax and in the vesicular fluid of two cases of human cutaneous anthrax (Cherry and Biegeleisen, *unpublished data*). The conjugate used was not specific for *B. anthracis* since it stained some encapsulated strains of *B. megaterium* and of *B. subtilis*, but when judiciously applied to appropriate material, it gave valuable information. For example, Biegeleisen (1*b*), using impression smears, obtained brilliant staining of *B. anthracis* in specimens of dried beef (jerky) used as food on an Indian reservation which experienced an outbreak of cutaneous anthrax and gastrointestinal disease after the consumption of meat from an infected steer. The staining was confirmed by isolation and identification of the anthrax bacillus. In another study (1*e*), fluores-

cein-labeled globulin for *B. anthracis* was used successfully to shorten the time required for both presumptive and definitive identification of the anthrax bacillus in environmental specimens from a goat-hair processing mill. Presumptive identification was based on the FA staining of colonies from blood-agar; definitive identification was based on application to impression smears of tissues from infected mice.

Recently, Franek (27*a*) used fluorescein-labeled anticapsular globulin for *B. anthracis* to study the pathogenesis of anthrax in guinea pigs and mice experimentally infected with spore suspensions. He confirmed the findings of earlier workers (5*b*) in regard to the value of this procedure for the identification of anthrax bacilli in tissue imprints, although he questioned the reliability of the test when applied to the detection of diffuse antigen in tissues. Franek described the appearance, chiefly in spleen cells, of fluorescent intracellular granules during the first few hours of the infection and of rods with developing capsules during the latter stages of the infectious process. He offered convincing evidence that these granules were derived from the bacterial capsules as a result of host defense mechanisms. The rapidity of the FA procedure and its advantages as compared with the usual staining techniques were emphasized.

Conjugates for *B. anthracis* can be used for obtaining valuable diagnostic information rapidly, and this knowledge can serve as a guide to the treatment of cutaneous anthrax in man. It also may aid in the diagnosis and treatment of anthrax in the lower animals. FA reagents can be used successfully for retrospective diagnosis of this disease when applied to the study of sectioned tissue.

Leptospira. Although Sheldon (69*a*) demonstrated *L. icterohaemorrhagiae* antigen in human muscle lesions with the FA technique as early as 1953, no further work appeared until 1957. At that time, Moulton and Howarth (50*a*) showed that *L. canicola* could be stained specifically in frozen sections of hamster kidney tissue that had been fixed in acetone. They also stained *Leptospira* cells in impression smears of Millipore filter membranes through which cultures of the organisms had been passed. White and Ristic (89*c*) used the FA procedure to stain *L. pomona* in ethyl alcohol-fixed impression smears of guinea pig kidney and in smears of the sediment of centrifuged guinea pig and bovine urine fixed in the same way. They concluded that the FA test provided a practical and efficient means of detecting *Leptospira* in urine from animals shedding the organisms. They were unsuccessful in demonstrating *Leptospira* in smears of the plasma of infected animals. The conjugate for *L. pomona* cross-reacted with other species of *Leptospira*. Other workers (3*a*) conducted a more extensive comparison of various techniques for the detection of *L. pomona* in both naturally and experimentally infected animal urine and tissues. The efficiency of the FA-staining method proved to be somewhat less than that of cultural methods, but was thought to be quite useful for examination of urine specimens contaminated with fast-growing organisms. No difficulty was experienced in recognizing the organisms by immunofluorescence when the fluids or tissues contained them in large numbers. Smears prepared from cultures or from urine were fixed in absolute ethyl alcohol, and tissue-impression smears were fixed in acetone at −15 to −20 C. Subsequently, White, Stoliker, and Galton (89*d*), using the FA test, examined ethyl alcohol-fixed smears of the urine and tissues of dogs naturally infected with leptospires. They found the FA test to be somewhat less reliable than cultural procedures on urine obtained by aseptic bladder tap, especially when small numbers of organisms were present.

The development of improved methods for the processing of formalin-fixed tissues which are to be subjected to immunofluorescence studies has extended the range of the technique (6*a*, 42*a*, 42*b*). Coffin and Maestrone (6*a*) froze formalin-preserved tissue and cut sections in a cryostat. The sections were fixed in acetone and then in graded concentrations of ethyl alcohol or methanol. Ethyl alcohol at a concentration of 70% either removes or denatures the surface antigen of *Leptospira* species (42*b*). Coffin and Maestrone used the direct, indirect, and complement-staining FA procedures to demonstrate leptospires in culture and in the tissues and fluids of embryonated eggs and infected dogs. It was found that the failure of fluorescein-labeled conjugates to react with antigens in tissues preserved with 10% formalin could be reversed by treating the sections or smears with a 1% solution of ammonium hydroxide just prior to staining. Maestrone (42*b*) expanded the investigation to the study of tissues from guinea pigs, dogs, hamsters, horses, cattle, swine, and feral animals. Major technical improvements consisted of the following: (i) confirmation of the efficacy of ammonium hydroxide in reversing the formalin inhibition of FA staining, (ii) a fourfold increase in the brightness of staining obtained by pretreatment of the preparation with a 3% solution of a surfactant, and (iii) the application of a rhodamine counterstain after the immune conjugate.

When the above procedures were used, the sensitivity of the FA test on both fresh and formalin-fixed tissues compared very favorably with that obtained by other investigators using serological, cultural, animal inoculation, and silver

impregnation techniques on the same specimens. The use of formalin-fixed tissues allows greater flexibility in the handling of field specimens. Furthermore, the FA examination can be performed quickly, and valid results can be obtained from contaminated specimens or from those in which the leptospires are no longer viable.

Reports on the serotyping of *Leptospira* with FA techniques and on the detection of serum antibody by use of the indirect FA test leave much to be desired. For the present, it is probably accurate to say that these are not reliable procedures. A summary of diagnostic applications is presented in Table 4.

Listeria monocytogenes. Difficulties in the isolation of *L. monocytogenes* and the frequent need for retrospective diagnosis of listeriosis by examination of formalin-fixed tissues has stimulated an interest in the employment of FA techniques for this purpose. Smith, Marshall, and Eveland (70*a*) prepared and tested a polyvalent, somatic, fluorescein-labeled antiserum that stained all of 30 strains of *Listeria* and did not react with any of the 180 strains of heterologous species. Their results are difficult to interpret, since the work was done with labeled whole sera which would be expected to contain serum protein fractions of widely different F:P ratios, and since the serological contributions of the somatic versus the flagellar antibodies were not clarified. They stated that 25 sera from persons with no history of listeric infection gave specific immunofluorescence in the indirect test just as did the sera from five patients with clinical disease. They also suggested the possibility of detecting species of *Listeria* in formalin-fixed tissue. Subsequently, Smith and Metzger (70*b*) showed that *Listeria* cells could be stained successfully in sections prepared from paraffin-embedded tissues which had been fixed in 10% formalin and processed in the usual way. They discussed the addition of guinea pig complement to the conjugate to increase both the brilliance and persistence of fluorescence. Good results have been obtained without this procedure by workers using γ-globulin serum fractions rather than labeled whole serum. Likewise, *L. monocytogenes* in tissues can be detected reliably (1*a*, 84*a*) without the use of bovine serum albumin labeled with Lissamine rhodamine RB 200 as a counterstain, as recommended by Smith and Metzger.

Conjugates for *L. monocytogenes* have proved valuable in the retrospective diagnosis of several cases of infection apparently due to this organism (1*a*, 84*a*). Type 4a was specifically stained in sections of formalin-fixed, paraffin-embedded tissue from a case that was clinically typical of listeria bacteremia of the new-born (84*a*). Biegeleisen (1*a*, and *unpublished data*) stained *Listeria* cells in sections of tissue from an aborted human fetus and from two cases of bacteremia of the new-born. He also detected this organism in smears from a human brain abscess and from a bovine brain. In none of these five cases was the type identification made with certainty, because a polyvalent conjugate that stained types 1, 2, and 3 was used. However, type 1 is the only one of the three types that is common in the United States. Biegeleisen (*unpublished data*) also showed that all serotypes of *Listeria* were stained brilliantly and specifically in sections of formalin-fixed, paraffin-embedded tissues of experimentally infected mice. There is no doubt that the antigens of these organisms are quite stable under such conditions.

Eveland (26) identified *Listeria* cells in a smear of the sediment of human cerebrospinal fluid by using a polyvalent FA conjugate. FA reagents have been used for the screening of vaginal secretions of pregnant women in an attempt to identify those whose flora may include *L. monocytogenes* (43*f*). Fluorescing organisms were observed one or more times in 22 of 496 pregnant women, and the majority of the positive reactions were obtained with type 1 conjugate. Since no isolations were made, the significance of these results is unknown. A summary of the above application is given in Table 4.

PROBLEMS AND POSSIBLE SOLUTIONS

The two most important deterrents to the more widespread use of the FA technique in the public health laboratory are equipment problems and the inadequacy of commercial FA reagents. The major equipment problem is the failure of the user to obtain consistently the expected 200 hr of life from the Osram HBO-200 mercury-arc lighting source. Too frequently this failure is coupled with a lamp explosion that usually shatters the reflecting mirror and often pits or cracks the collecting lens of the optical system. Thus, significant expense beyond that of lamp replacement is incurred. The Osram lamp is mentioned only because this lamp is used in the vast majority of FA equipment in use in the United States today.

Improper operating conditions and defects in the design of lamp housings are important factors in lamp failure. Insuring proper operating conditions is the joint responsibility of the manufacturer of FA lighting equipment and of the customer; housing design is the responsibility of the manufacturer. The extent to which lamp failure is the result of defective manufacture is difficult to determine, but it is judged to be a factor of some importance. The former problems are being defined by monitoring the power input to the lamp with a voltmeter and ammeter with con-

comitant adjustment to give the desired operating conditions. Some manufacturers are supplying either a-c or d-c voltage stabilized power supplies which, if properly designed, eliminate problems due to variable voltage from the supply mains. In some equipment under certain conditions, there appears to be local overheating of the cathode of the mercury-arc lamp, leading to corrosion of the connecting copper wire and to the production of strain in the quartz envelope of the lamp. Overheating may seriously reduce the effective lamp life, or the lamp may fail suddenly due either to the development of a crack in the quartz lamp stem or to explosion. Modifications, such as heat sinks and the substitution of noncorrosive nickel for copper wire for the cathode connection, are being devised by the manufacturers. There is every reason to hope that these devices will substantially extend effective lamp life. There are many units of FA equipment, produced by at least three of the leading manufacturers, that have given excellent service over a period of several years of constant use without significant problems of any kind.

The advent of a domestic supply of mercury-arc lamps that can be substituted successfully for the Osram lamp hopefully may be expected to improve the position of the user by enhancing quality through competition and by providing a more convenient basis for collaborative effort to improve lamp performance. Domestic lamps of this type are now available, but further discussion must await widespread evaluation of their performance.

Problems regarding commercial FA reagents in bacteriology continue to frustrate the laboratory worker, although, upon investigation, it sometimes is found that a perfectly satisfactory reagent is being misused by the purchaser. Nevertheless, commercial FA reagents may be inadequate and are sometimes useless. Competition forces manufacturers to market conjugates diluted almost to titer. This is detrimental to their stability. It also prohibits adjustment of concentration to compensate for decreased potency. Some manufacturers are careless about determining the specificity of their products or fail to perform the sorptions necessary to impart the desired degree of reliability. Too often, not enough descriptive data on the preparation and use of the conjugate is furnished to the purchaser to permit him to interpret results with reasonable accuracy. At times, FA reagents and procedures are recommended by the manufacturer for use on diagnostic specimens in the absence of supporting data indicating their reliability.

These problems are common to all diagnostic reagents, but acutely so to FA reagents owing to lack of experience and guide lines. It appears that no real progress can be made in this area until all major producers accept and adhere to established standards for production and testing of each reagent and submit their products for evaluation and certification by an independent agency.

SUMMARY

An attempt has been made in this review to present a critical evaluation of the current status of immunofluorescence as a technique for the diagnosis of bacterial infections in the public health laboratories. As with other new techniques, the specific tests that succeed must offer better diagnostic service without increased cost, or diagnostic service which is equal in reliability to that given by other tests but at reduced cost. Insofar as can be determined without detailed cost analyses, it appears that some of the applications discussed in this review have fulfilled the above criteria, and that the others may do so in the future. Outstanding among the former are the applications of the FA test to the identification of group A streptococci in nasopharyngeal exudates, the serogrouping of the enteropathogenic *E. coli* in fecal smears, the identification of the gonococcus in exudates from the reproductive tract, and the use of the indirect FA test to detect the presence of syphilitic antibody in human sera. Less advanced are applications of immunofluorescence to the detection of the typhoid bacillus in carriers or cases, the identification of the whooping cough bacillus in specimens from the nasopharynx, and the screening of spinal-fluid sediments for the common etiological agents of bacterial meningitis. FA reagents aid the clinician in making a rapid presumptive diagnosis of diphtheria in acute cases where a decision regarding therapy must be made without delay. Future development and evaluation will determine the usefulness of fluorescein-labeled antibody for the grouping of *Shigella* species in fecal smears.

Some examples of the massive use of FA tests in certain diagnostic public health laboratories were given. The figures cited were obtained from only five state health laboratories; therefore, they do not reflect the widespread use of immunofluorescent techniques in diagnostic work.

Two major problems impeding implementation of immunofluorescence in diagnostic work are those of unsatisfactory life of the mercury-arc lighting source and the scarcity of reliable diagnostic reagents. Both of these problems are soluble, and considerable progress is being made.

LITERATURE CITED

1. ALLEN, J. C., AND L. E. CLUFF. 1963. Identification of toxinogenic *C. diphtheriae*

with fluorescent antitoxin: demonstration of its nonspecificity. Proc. Soc. Exptl. Biol. Med. **112**:194–199.

1*a*. BIEGELEISEN, J. Z. 1964. Immunofluorescence techniques in retrospective diagnosis of human listeriosis. J. Bacteriol. **87**:1257–1258.

1*b*. BIEGELEISEN, J. Z. 1964. Immunofluorescent staining of *Bacillus anthracis* in dried beef. J. Bacteriol. **88**:260–261.

1*c*. BIEGELEISEN, J. Z., M. D. MOODY, B. B. MARCUS, AND J. W. FLYNT. 1962. The use of fluorescein-labeled anti-*Brucella suis* globulin for demonstrating *Brucella* antigen in animal tissues. Am. J. Vet. Res. **23**:592–595.

1*d*. BIEGELEISEN, J. Z., B. B. BRADSHAW, AND M. D. MOODY. 1962. Demonstration of *Brucella* antibodies in human serum. J. Immunol. **88**:109–112.

1*e*. BIEGELEISEN, J. Z., W. B. CHERRY, P. SKALIY, AND M. D. MOODY. 1962. The demonstration of *Bacillus anthracis* in environmental specimens by conventional and fluorescent antibody techniques. Am. J. Hyg. **75**:230–239.

1*f*. BIEGELEISEN, J. Z., B. B. MARCUS, D. L. RHODEN, AND L. N. GIRARD. 1964. Immunofluorescent staining of *Listeria monocytogenes* in smears prepared from artificial mediums, cerebrospinal fluid and murine tissue. Health Lab. Sci. **1**:273–278.

2. BOREL, L. J., AND P. DUREL. 1959. L'immunofluorescence appliquee au diagnostic de la syphilis. Pathol. Biol. Semaine Hop. **7**: 2317–2324.

3. BORIS, M., B. M. THOMASON, V. D. HINES, T. S. MONTAGUE, AND T. F. SELLERS. 1964. A community epidemic of enteropathogenic *Escherichia coli* O126:B16:NM gastroenteritis associated with asymptomatic respiratory infection. Pediatrics **33**:18–29.

3*a*. BOULANGER, P., AND A. ROBERTSON. 1961. Fluorescein-labeled antibody technique for the demonstration of *Leptospira pomona*. Can. J. Comp. Med. Vet. Sci. **25**: 299–306.

4. BROWN, L., M. B. COPELOFF, AND W. L. PEACOCK. 1962. Study of gonorrhea in treated and untreated asymptomatic women as determined by fluorescent antibody and culture methods. Am. J. Obstet. Gynecol. **84**:753–757.

4*a*. CAVANAUGH, D. C., AND R. RANDALL. 1959. The role of multiplication of *Pasteurella pestis* in mononuclear phagocytes in the pathogenesis of flea-borne plague. J. Immunol. **83**:348–363.

5. CENSUALES, S., AND V. GAROFALO. 1959. Anticorpi antitreponemici nella sifilide umana svelati con la reazione di fluorescenza. Riv. Ist. Sieroterap. Ital. **34**:161–167.

5*b*. CHERRY, W. B., AND E. M. FREEMAN. 1959. Staining bacterial smears with fluorescent antibody. Zentr. Bakteriol. Parasitenk. Abt. I Orig. **175**:582–604.

6. CHERRY, W. B., B. M. THOMASON, A. POMALES-LEBRÓN, AND W. H. EWING. 1961. Rapid presumptive identification of enteropathogenic *Escherichia coli* in fecal smears by means of fluorescent antibody. Bull. World Health Organ. **25**:159–171.

6*a*. COFFIN, D. L., AND G. MAESTRONE. 1962. Detection of leptospires by fluorescent antibody. Am. J. Vet. Res. **23**:159–164.

7. COHEN, F., R. H. PAGE, AND C. S. STULBERG. 1961. Immunofluorescence in diagnostic bacteriology. III. The identification of enteropathogenic *E. coli* serotypes in fecal smears. Am. J. Diseases Children **102**: 82–90.

8. COHEN, J. O., G. S. COWART, AND W. B. CHERRY. 1961. Antibodies against *Staphylococcus aureus* in nonimmunized rabbits. J. Bacteriol. **82**:110–114.

9. COHEN, J. O., W. L. NEWTON, W. B. CHERRY, AND E. L. UPDYKE. 1963. Normally occurring staphylococcal antibodies in germfree mice. J. Immunol. **90**:358–367.

10. COONS, A. H., H. J. CREECH, AND R. N. JONES. 1941. Immunological properties of an antibody containing a fluorescent group. Proc. Soc. Exptl. Biol. Med. **47**:200–202.

11. COONS, A. H., H. J. CREECH, R. N. JONES, AND E. BERLINER. 1942. The demonstration of pneumococcal antigen in tissues by the use of fluorescent antibody. J. Immunol. **45**:159–170.

12. COONS, A. H., AND M. H. KAPLAN. 1950. Localization of antigen in tissue cells. II. Improvement in methods for detection of antigen by means of fluorescent antibody. J. Exptl. Med. **91**:1–13.

13. DAGUET, G. L., AND J. PILLOT. 1962. Sérologie de la syphilis. Anticorps responsables de l'immunofluorescence, et antiprotéine de groupe dans les sérums donnant des réactions cardiolipidiques foussement positives. Ann. Inst. Pasteur **102**:364–366.

14. DANIELSSON, D., AND G. LAURELL. 1961. Identification of enteropathogenic *Escherichia coli* O111:B4 by means of fluorescent antibodies. Acta Paediat. **50**:339–345.

15. DANIELSSON, D. 1963. The demonstration of *N. gonorrhoeae* with the aid of fluorescent antibodies. 1. Immunological studies of antigonococcal sera and their fluorescein-labelled globulins, with particular regard to specificity. Acta Dermato-Venereol. **43**:451–464.

16. DANIELSSON, D. 1963. The demonstration of *N. gonorrhoeae* with the aid of fluorescent antibodies. 2. A comparison between the fluorescent antibody technique and conventional methods of detecting *N. gonor-*

rhoeae in men and women. Acta Dermato-Venereol. **43**:511–521.

17. DEACON, W. E., V. H. FALCONE, AND A. HARRIS. 1957. A fluorescent test for treponemal antibody. Proc. Soc. Exptl. Biol. Med. **96**:477–480.
18. DEACON, W. E., W. L. PEACOCK, JR., E. M. FREEMAN, AND A. HARRIS. 1959. Identification of *Neisseria gonorrhoeae* by means of fluorescent antibodies. Proc. Soc. Exptl. Biol. Med. **101**:322–325.
19. DEACON, W. E., E. M. FREEMAN, AND A. HARRIS. 1960. Fluorescent treponemal antibody test. Modification based on quantitation (FTA-200). Proc. Soc. Exptl. Biol. Med. **103**:827–829.
20. DEACON, W. E., W. L. PEACOCK, E. M. FREEMAN, A. HARRIS, AND W. L. BUNCH. 1960. Fluorescent antibody tests for detection of the gonococcus in women. Public Health Rept. U.S. **75**:125–129.
21. DEACON, W. E. 1961. Fluorescent antibody methods for *Neisseria gonorrhoeae* identification. Bull. World Health Organ. **24**: 349–355.
22. DEACON, W. E., AND E. F. HUNTER. 1962. Treponemal antigens as related to identification and syphilis serology. Proc. Soc. Exptl. Biol. Med. **110**:352–356.
23. DONALDSON, P., AND J. A. WHITAKER. 1960. Diagnosis of pertussis by fluorescent antibody staining of nasopharyngeal smears. A.M.A. J. Diseases Children **99**:423–427.
24. EDWARDS, E. A. 1962. Detecting *Treponema pallidum* in primary lesions by the fluorescent antibody technique. Public Health Rept. U.S. **77**:427–430.
25. ELDERING, G., W. C. EVELAND, AND P. L. KENDRICK. 1962. Fluorescent antibody staining and agglutination reactions in *Bordetella pertussis* cultures. J. Bacteriol. **83**:745–749.
26. EVELAND, W. C. 1963. Demonstration of *Listeria monocytogenes* in direct examination of spinal fluid by fluorescent-antibody technique. J. Bacteriol. **85**:1448–1450.
27. FIFE, E. H., JR., B. M. BRYAN, R. W. SANDERS, AND L. H. MUSCHEL. 1961. Evaluation of the fluorescent treponemal antibody (FTA) test for syphilis. Comparison with *Treponema pallidum* immobilization (TPI) test. Am. J. Clin. Pathol. **36**:105–113.

27*a*. FRANEK, J. 1964. Application of fluorescent antibodies for demonstrating *B. anthracis* in the organs of infected animals. J. Hyg. Epidemiol. Microbiol. Immunol. (Prague) **8**:111–119.

28. Gonococcus. Procedures for isolation and identification. U.S. Public Health Serv. Publ. **499** (Rev. 1962).
29. GROSSMAN, M., S. SUSSMAN, D. GOTTFRIED C. QUOCK, AND W. TICKNOR. 1964. Immunofluorescent techniques in bacterial meningitis. Am. J. Diseases Children **107**:356–362.
30. HARRIS, A., H. M. BOSSAK, W. E. DEACON, AND W. L. BUNCH. 1960. Comparison of the fluorescent treponemal antibody test with other tests for syphilis on cerebrospinal fluids. Brit. J. Venereal Diseases **36**:178–180.
31. HARRIS, A., W. E. DEACON, J. TIEDEMANN, AND W. L. PEACOCK. 1961. Fluorescent antibody method of detecting gonorrhea in asymptomatic females. Public Health Rept. U.S. **76**:93–96.
32. HOLWERDA, J., AND G. ELDERING. 1963. Culture and fluorescent-antibody methods in diagnosis of whooping cough. J. Bacteriol. **86**:449–451.

32*a*. HUDSON, B. W., S. F. QUAN, AND L. KARTMAN. 1962. Efficacy of fluorescent antibody methods for detection of *Pasteurella pestis* in carcasses of albino laboratory mice stored for various periods. J. Hyg. **60**: 443–450.

33. HUNTER, E. F., W. E. DEACON, AND P. E. MEYER. 1964. An improved FTA test for syphilis, the absorption procedure (FTA-ABS). Public Health Rept. U.S. **79**:410–412.

33*a*. JAEGER, R. F., R. O. SPERTZEL, AND R. W. KUEHNE. 1961. Detection of air-borne *Pasteurella tularensis* using the fluorescent antibody technique. Appl. Microbiol. **9**:585–587.

33*b*. JANNEY, G. C., AND D. T. BERMAN. 1961. Staining intracellular Brucella organisms by means of fluorescent antibodies. Am. J. Vet. Res. **23**:596–598.

34. JONES, W. L., AND M. D. MOODY. 1960. Staining toxigenic *Corynebacterium diphtheriae* with fluorescent antitoxin. Bacteriol. Proc., p. 141.

34*a*. KARAKAWA, W. W., E. K. BORMAN, AND C. R. MCFARLAND. 1964. Typing of group A streptococci by immunofluorescence. I. Preparation and properties of type 1 fluorescein-labeled antibody. J. Bacteriol. **87**:1377–1382.

34*b*. KARTMAN, L. 1960. The role of rabbits in sylvatic plague epidemiology, with special attention to human cases in New Mexico and use of the fluorescent antibody technique for detection of *Pasteurella pestis* in field specimens. Zoonoses Res. **1**:1–27.

35. KELLOGG, D. S., AND W. E. DEACON. 1964. A new rapid immunofluorescent staining technique for identification of *Treponema pallidum* and *Neisseria gonorrhoeae*. Proc. Soc. Exptl. Biol. Med. **115**:963–965.
36. KENDRICK, P. L., G. ELDERING, AND W. C. EVELAND. 1961. Fluorescent antibody techniques. Methods for identification of *Bordetella pertussis*. A.M.A. J. Diseases Children **101**:149–154.
37. KENT, J. F., S. V. COVERT, H. W. REILLY, W. H. KINCH, AND W. B. LAWSON. 1962. Evaluation of fluorescent treponemal anti-

body (RFTA and FTA II) and other tests (RPCF and TPI) for syphilis. Proc. Soc. Exptl. Biol. Med. **109**:584–589.

38. LABREC, E. H., S. B. FORMAL, AND H. SCHNEIDER. 1959. Serological identification of *Shigella flexneri* by means of fluorescent antibody. J. Bacteriol. **78**:384–391.
39. LAUTROP, H. 1950. Studies on the antigenic structure of *Corynebacterium diphtheriae*. Acta Pathol. Microbiol. Scand. **27**:443–447.
40. LEIBOVITZ, A., T. R. OBERHOFER, J. T. MEACHAM, AND T. N. DIESTELHORST. 1963. Enhancement of specificity of the fluorescent treponemal antibody test as compared with the TPI test. Am. J. Clin. Pathol. **40**:480–486.
41. LEVINA, E. N. 1958. Fluorescein-labeled antibody for the detection of the anthrax bacillus. J. Microbiol. Epidemiol. Immunobiol. (USSR) Engl. Transl. **29**:6–11.
42. LIND, I. 1962. Identification of *Neisseria gonorrhoeae* by fluorescent antibody techniques. WHO/VDT/295. Expert Committee on Gonococcal Infections.

42*a*. MAESTRONE, G. 1963. Demonstration of leptospiral and viral antigens in formalin-fixed tissues. Nature **197**:409–410.

42*b*. MAESTRONE, G. 1963. The use of an improved fluorescent antibody procedure in the demonstration of *Leptospira* in animal tissues. Can. J. Comp. Med. Vet. Sci. **27**:108–112.

43. MARIE, J., G. KERZOG, AND M. GAIFFE. 1963. Le diagnostic rapide de la coqueluche par la technique des anticorps fluorescents (100 premiers resultants). Ann. Pediat. Semaine Hop. **10**:53–56.

43*a*. MCCAHAN, G. R., M. D. MOODY, AND F. A. HAYES. 1962. An epizootic of tularemia among rabbits in Northwestern South Carolina. Am. J. Hyg. **75**:335–338.

43*b*. MCGAVRAN, M. H., J. D. WHITE, H. T. EIGELSBACH, AND R. W. KERPSACK. 1962. Morphologic and immunohistochemical studies of the pathogenesis of infection and antibody formation subsequent to vaccination of *Macaca irus* with an attenuated strain of *Pasteurella tularensis* I. Intracutaneous vaccination. Am. J. Pathol. **41**:259–271.

43*c*. MCKEEVER, S., J. H. SCHUBERT, M. D. MOODY, G. W. GORMAN, AND J. F. CHAPMAN. 1958. Natural occurrence of tularemia in marsupials, carnivores, lagomorphs, and large rodents in Southwestern Georgia and Northwestern Florida. J. Infect. Diseases **103**:120–126.

43*d*. MERRIOTT, J., A. SHOEMAKER, AND C. M. DOWNS. 1961. Growth of *Pasteurella tularensis* in cultured cells. J. Infect. Diseases **108**:136–150.

43*e*. METZGER, J. F., AND C. W. SMITH. 1960. Rapid identification of *Neisseria meningitidis* by fluorescent antibody technique. U.S. Armed Forces Med. J. **11**:1185–1189.

43*f*. MILLER, J. K., AND T. MURASCHI. 1963. Relation of *Listeria monocytogenes* in vaginal flora as detected by immunofluorescence and the interruption of pregnancy. Symp. on Listeric Infection, 2nd, Montana State College, Bozeman.

44. MONTGOMERY, C. H., S. SUHRLAND, AND J. M. KNOX. 1960. Observations concerning fluorescent treponemal antibody test for syphilis. J. Invest. Dermatol. **35**:95–101.
45. MOODY, M. D., M. GOLDMAN, AND B. M. THOMASON. 1956. Staining bacterial smears with fluorescent antibody. I. General methods for *Malleomyces pseudomallei*. J. Bacteriol. **72**:357–361.
46. MOODY, M. D., E. C. ELLIS, AND E. L. UPDYKE. 1958. Staining bacterial smears with fluorescent antibody. IV. Grouping streptococci with fluorescent antibody. J. Bacteriol. **75**:553–560.

46*a*. MOODY, M. D., B. M. THOMASON, C. C. WINTER, AND A. D. HALL. 1959. Sensitivity of bacterial agglutination and fluorescent antibody reactions. Bacteriol. Proc., p. 89.

46*b*. MOODY, M. D., AND C. C. WINTER. 1959. Rapid identification of *Pasteurella pestis* with fluorescent antibody. III. Staining *Pasteurella pestis* in tissue impression smears. J. Infect. Diseases **104**:288–294.

47. MOODY, M. D., AND W. L. JONES. 1960. Differentiation of diphtheria and diphtheroid bacilli with fluorescent antibody. Bacteriol. Proc., p. 145–146.

47*a*. MOODY, M. D., J. Z. BIEGELEISEN, JR., AND G. C. TAYLOR. 1961. Detection of brucellae and their antibodies by fluorescent antibody and agglutination tests. J. Bacteriol. **81**:990–995.

48. MOODY, M. D., AND W. L. JONES. 1963. Identification of *Corynebacterium diphtheriae* with fluorescent antibacterial reagents. J. Bacteriol. **86**:285–293.
49. MOODY, M. D., A. C. SIEGEL, B. PITTMAN, AND C. C. WINTER. 1963. Fluorescent-antibody identification of Group A streptococci from throat swabs. Am. J. Public Health **53**:1083–1092.
50. MOORE, M. B., JR., E. M. VANDERSTOEP, R. D. WENDE, AND J. M. KNOX. 1963. Fluorescent gonococcal antibody technique in gonorrhea in the male. Public Health Rept. U.S. **78**:90–92.

50*a*. MOULTON, J. E., AND J. A. HOWARTH. 1957. The demonstration of *Leptospira canicola* in hamster kidneys by means of fluorescent antibody. Cornell Vet. **47**:524–532.

50*b*. MOULTON, J. E., AND M. E. MEYER. 1958. The pathogenesis of *Brucella suis* infection in guinea pigs. Lesions of the spleen, liver, testis, and articulations. Cornell Vet. **48**:165–195.

51. MYERBURG, R. J., J. M. JABLON, J. A. MAZ-

ZARELLA, AND M. S. SASLAW. 1964. Evaluation of FA and conventional techniques for identifying group A beta hemolytic streptococci. Public Health Rept. U.S. **79**:510–514.

52. NIEL, G., AND A. FRIBOURG-BLANC. 1962. Technique actuelle du test d'immunofluorescence appliqué au diagnostic de la syphilis. Ann. Inst. Pasteur **102**:616–628.
53. NIELSEN, H. A., AND O. IDSØE. 1962. Evaluation of the fluorescent treponemal antibody test (FTA). WHO/VDT/RES/25—WHO/VDT/SERO/102.
54. NELSON, J. D., J. A. WHITAKER, AND B. HEMPSTEAD. 1960. Diagnosis of enteropathogenic *E. coli* diarrhea by fluorescein-labeled antibodies. J. Pediat. **57**:684–688.
55. NELSON, J. D., J. A. WHITAKER, B. HEMPSTEAD, AND M. HARRIS. 1961. Epidemiological application of the fluorescent antibody technique. J. Am. Med. Assoc. **176**:26–30.
56. OLANSKY, S., AND G. F. MCCORMICK. 1960. Experiences with the fluorescent treponemal antibody test for syphilis (FTA Test). A.M.A. Arch. Dermatol. **81**:59–65.
57. PAGE, R. H., G. L. CALDRONEY, AND C. S. STULBERG. 1961. Immunofluorescence in diagnostic bacteriology. I. Direct identification of *Hemophilis influenzae* in smears of cerebrospinal fluid sediments. Am. J. Diseases Children **101**:155–159.
58. PAGE, R. H., AND C. S. STULBERG. 1962. Immunofluorescence in epidemiologic control of *E. coli* diarrhea. Am. J. Diseases Children **104**:149–156.
59. PEEPLES, W. J., D. W. SPIELMAN, AND M. D. MOODY. 1961. Field application of fluorescent antibody technique for identification of Group A streptococcus. Public Health Rept. U.S. **76**:651–654.
60. PITTMAN, B., AND M. D. MOODY. 1960. Staining of *Staphylococcus aureus* with fluorescein-labeled globulin from non-immunized and streptococcus-immunized animals. Bacteriol. Proc., p. 140.
61. PRICE, E. V. 1964. Field evaluation of fluorescent antibody technique in the detection of the gonococcus in the female. Ann. Symp. Recent Advan. Study Venereal Diseases, 14th, Houston, Tex.
62. PUCCINELLI, V. 1961. Il valore ed il significato dello FTAT (Fluorescent Treponemal Antibody Test) nella sierologia della sifilide. Arcispedale S. Anna Ferrara **14**:781–788.
63. REDYS, J. J., M. R. ROSS, AND E. K. BORMAN. 1960. Inhibition of common antigen fluorescence in grouping streptococci by the fluorescent antibody method. J. Bacteriol. **80**:823–829.
64. REDYS, J. J., A. B. PARZICK, AND E. K. BORMAN. 1963. Detection of group A streptococci in throat cultures by immunofluorescence. Public Health Rept. U.S. **78**:222–226.

64*a*. RIGGS, J. L., R. J. SEIWALD, J. H. BURCKHALTER, C. M. DOWNS, AND T. G. METCALF. 1958. Isothiocyanate compounds as fluorescent labeling agents for immune serum. Am. J. Pathol. **34**:1081–1097.

64*b*. SCARBOROUGH, L. C., JR. 1963. A study of laboratory methods for the detection of *Brucella* in blood by fluorescent antibody. M.S. Thesis, State Univ. of Iowa, Iowa City.

65. SCOTT, E. G., AND W. J. HOLLOWAY. 1963. Identification of Group A streptococci in throat cultures by the fluorescent antibody technique. Delaware Med. J. **35**:34–36.
66. SELL, S. H., W. J. CHEATHAM, B. YOUNG, AND K. WELCH. 1963. *Hemophilus influenzae* in respiratory infections. I. Typing by immunofluorescent techniques. Am. J. Diseases Children **105**:466–469.
67. SELL, S. H., R. S. SANDERS, AND W. J. CHEATHAM. 1963. *Hemophilus influenzae* in respiratory infections. II. Specific serological antibodies identified by agglutination and immunofluorescent techniques. Am. J. Diseases Children **105**:470–474.
68. Serology Evaluation and Research Assembly 1956–1957. 1959. U.S. Public Health Serv. Publ. **650**.
69. SHAUGHNESSY, H. J., M. LESKO, F. DORIGAN, G. F. FORSTER, R. A. MORRISSEY, AND D. M. KESSNER. 1962. An extensive community outbreak of diarrhea due to enteropathogenic *Escherichia coli* O111:B4. Am. J. Hyg. **76**:44–51.

69*a*. SHELDON, W. H. 1953. Leptospiral antigen demonstrated by the fluorescent antibody technique in human muscle lesions of *Leptospira icterohaemorrhagiae*. Proc. Soc. Exptl. Biol. Med. **84**:165–167.

70. SIBOULET, A., A. FRIBOURG-BLANC, AND G. NIEL. 1963. Application pratique de la technique d'immuno-fluorescence au diagnostic sérologique de la syphilis; á propos de cent cas cliniques. Bull. Soc. Franc. Dermatol. Syphilig. **69**:892–897.

70*a*. SMITH, C. W., J. D. MARSHALL, JR., AND W. C. EVELAND. 1960. Identification of *Listeria monocytogenes* by the fluorescent antibody technic. Proc. Soc. Exptl. Biol. Med. **103**:842–845.

70*b*. SMITH, C. W., AND J. F. METZGER. 1963. Identification of *Listeria monocytogenes* in experimentally infected animal tissue by immunofluorescence. Symp. Listeric Infection, 2nd, Montana State College, Bozeman.

70*c*. SMITH, C. W., J. F. METZGER, AND M. D. HOGGAN. 1962. Immunofluorescence as applied to pathology. Am. J. Clin. Pathol. **38**:26–42.

71. SPAGNOLI, U., AND E. MANNUCCI. 1960. Primi

risultati con la reazione di fluorescenza (FTA) applicata alla serodiagnosi della sifilide. Rass. Dermatol. Sifilog. **13**:323–336.

72. STILLERMAN, M., AND S. H. BERNSTEIN. 1961. Streptococcal pharyngitis. Evaluation of clinical syndromes in diagnosis. Am. J. Diseases Children **101**:476–489.
73. STOUT, G. W., V. H. FALCONE, AND M. B. MOORE, JR. 1964. Protocols for testing reagents employed in serologic tests for syphilis. Health Lab. Sci. **1**:119–127.
74. TAYLOR, C. E. D., D. J. LEA, G. V. HEIMER, AND A. J. H. TOMLINSON. 1963. Fluorescent antibody techniques in diagnostic bacteriology. Proc. Roy. Soc. Med. **56**:478.

74*a*. TAYLOR, C. E. D., AND G. V. HEIMER. 1964. Rapid diagnosis of Sonne dysentery by means of immunofluorescence. Brit. Med. J. **2**:165–166.

75. THIVOLET, J., AND D. CHERBY-GROSPIRON. 1961. Étude des facteurs de non-spécéficité des réactions d'immunofluorescence intérét dans la recherche des anticorps contre le tréponéme de la syphilis. Ann. Inst. Pasteur **101**:869–875.
76. THIVOLET, J., D. GROSPIRON, AND M. MURAT. 1960. Utilisation de l'immunofluorescence pour de diagnostic de la syphilis. Rev. Hyg. Med. Soc. Paris **8**:501–509.
77. THOMASON, B. M., M. D. MOODY, AND M. GOLDMAN. 1956. Staining bacterial smears with fluorescent antibody. II. Rapid detection of varying numbers of *Malleomyces pseudomallei* in contaminated materials and in infected animals. J. Bacteriol. **72**:362–367.
78. THOMASON, B. M., W. B. CHERRY, AND M. D. MOODY. 1957. Staining bacterial smears with fluorescent antibody. III. Antigenic analysis of *Salmonella typhosa* by means of fluorescent antibody and agglutination reactions. J. Bacteriol. **74**:525–532.
79. THOMASON, B. M., W. B. CHERRY, AND P. R. EDWARDS. 1959. Staining bacterial smears with fluorescent antibody. VI. Identification of salmonellae in fecal specimens. J. Bacteriol. **77**:478–486.
80. THOMASON, B. M., W. B. CHERRY, B. R. DAVIS, AND A. POMALES-LEBRÓN. 1961. Rapid presumptive identification of enteropathogenic *Escherichia coli* in fecal smears by means of fluorescent antibody. Bull. World Health Organ. **25**:137–152.
81. THOMASON, B. M., A. MCWHORTER, AND E. SANDERS. 1964. Rapid detection of typhoid carriers by means of fluorescent-antibody techniques. Bacteriol. Proc., p. 56.
82. TUCKER, C. B., G. M. CAMERON, R. L. BUCHANAN, A. DILLON, AND J. H. GRAYSON. 1962. An evaluation of the Kolmer Reiter protein and fluorescent treponemal antibody tests. Public Health Rept. U.S. **77**:1068–1094.

82*a*. U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE. Serologic tests for syphilis. U.S. Public Health Serv. Publ. **411** (Rev. May, 1964).

83. VAISMAN, A., A. HAMELIN, AND T. GUTHE. 1963. La technique des anticorps fluorescents pratiquée sur sang desséché et élué. Bull. World Health Organ. **29**:1–6.
84. VAISMAN, A., AND A. HAMELIN. 1961. Application au diagnostic sérologique de la syphilis de la méthode d'immunofluorescence. Presse Med. **69**:1157–1159.

84*a*. VILLELLA, R. L., L. W. HALLING, AND J. Z. BIEGELEISEN, JR. 1963. A case of listeriosis of the newborn with fluorescent antibody histologic studies. Am. J. Clin. Pathol. **40**:151–156.

85. WAGNER, M., AND H. HEINRICH. 1962. Sum fluoreszenz-serologischen Nachweis von Streptokokken der Gruppe A. Zentr. Bakteriol. Parasitenk. Abt. I **175**:582–604.

85*a*. WALKER, R. V. 1962. Studies on the immune response of guinea pigs to the envelope substance of *Pasteurella pestis*. II. Fluorescent antibody studies of cellular and tissue response in mice and guinea pigs to large doses of Fraction I. J. Immunol. **88**:164–173.

86. WARFIELD, M. A., R. H. PAGE, W. W. ZUELZER, AND C. S. STULBERG. 1961. Immunofluorescence in diagnostic bacteriology. II. Identification of Group A streptococci in throat smears. Am. J. Diseases Children **101**:160–163.
87. WHEELER, W. E. 1962. Where is our nosocomial conscience? Am. J. Diseases Children **104**:593–594.
88. WHITAKER, J. A., R. H. PAGE, C. S. STULBERG, AND W. W. ZUELZER. 1958. Rapid identification of enteropathogenic *Escherichia coli* O127:B8 by the fluorescent antibody technique. A.M.A. J. Diseases Children **95**:1–8.
89. WHITAKER, J. A., J. D. NELSON, AND C. W. FINK. 1961. The fluorescent antitoxin test for the immediate diagnosis of diphtheria. Pediatrics **27**:214–218.

89*a*. WHITE, J. D., AND G. P. BLUNDELL. 1958. The use of the fluorescent antibody technic for demonstration of *Pasteurella tularensis* in formalin-fixed tissues. Bacteriol. Proc., p. 136.

89*b*. WHITE, J. D., M. H. MCGAVRAN, P. A. PRICKETT, J. J. TULIS, AND H. T. EIGELSBACH. 1962. Morphologic and immunohistochemical studies of the pathogenesis of infection and antibody formation subsequent to vaccination of *Macaca irus* with an attenuated strain of *Pasteurella tularensis*. II. Aerogenic vaccination. Am. J. Pathol. **41**:405–413.

89*c*. WHITE, F. H., AND M. RISTIC. 1959. Detec-

tion of *Leptospira pomona* in guinea pig and bovine urine with fluorescein-labeled antibody. J. Infect. Diseases **105**:118–123.

89*d*. WHITE, F. H., H. E. STOLIKER, AND M. M. GALTON. 1961. Detection of leptospires in naturally infected dogs, using fluorescein-labeled antibody. Am. J. Vet. Res. **22**:650–654.

90. WILKINSON, A. E. 1961. Fluorescent treponemal antibody test. A preliminary report. Brit. J. Venereal Diseases **37**:59–63.

91. WILKINSON, A. E. 1963. The fluorescent treponemal antibody test in the serological diagnosis of syphilis. Proc. Roy. Soc. Med. **56**:478–481.

91*a*. WINTER, C. C., AND M. D. MOODY. 1959. Rapid identification of *Pasteurella pestis* with fluorescent antibody. II. Specific identification of *Pasteurella pestis* in dried smears. J. Infect. Diseases **104**:281–287.

92. WOLFE, M. D., AND G. M. CAMERON. 1959. Fluorescent antibody technique versus cultural methods as a routine procedure for finding beta-hemolytic streptococci in throat cultures. Public Health Lab. **17**:76–82.

93. WOLFE, M. D., G. M. CAMERON, AND M. E. WEST. 1960. Further observations on the fluorescent technique as a procedure for detecting group A streptococci in throat cultures. Public Health Lab. **18**:120–126.

93*a*. YAGER, R. H., R. O. SPERTZEL, R. F. JAEGER, AND W. D. TIGERTT. 1960. Domestic fowl-source of high titer *P. tularensis* serum for fluorescent antibody technic. Proc. Soc. Exptl. Biol. Med. **105**:651–654.

94. YOBS, A. R., L. BROWN, AND E. F. HUNTER. 1964. Fluorescent antibody technique in early syphilis. Arch. Pathol. **77**:220–225.